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A Role for Neuronal Nicotinic Acetylcholine Receptors in Dopamine-Mediated Behaviors and the Hypnotic Response to Anesthetics: A Dissertation

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A ROLE FOR NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS IN DOPAMINE-MEDIATED BEHAVIORS AND THE HYPNOTIC RESPONSE TO ANESTHETICS

A Dissertation Presented

By

LINDSEY G. SOLL

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NEUROSCIENCE

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ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels that most notably influence dopamine (DA) release. In this thesis, I examine the role of nAChRs in mediating DA-related behaviors such as movement and drug dependence. To accomplish this, I utilized a "gain-of-function" knock-in mouse (the Leu9'Ala line) containing agonist-hypersensitive α4* nAChRs (* indicates other nAChR subunits in addition to α4 are within the receptor complex) that renders receptors 50-fold more sensitive to nicotine and acetylcholine than wild-type (WT) receptors. I found that DHβE, a selective antagonist for α4β2* nAChRs, induced reversible and robust motor dysfunction characterized by hypolocomotion, akinesia, catalepsy, tremor, and clasping in Leu9'Ala but not WT mice. Reversal of the phenotype was achieved by targeting dopamine signaling. Blockade of mutant α4* nAChRs elicited activation of brain regions in the basal ganglia including dorsal striatum and substantia nigra pars reticulata indicated by c-Fos immunoreactivity. These data indicate that blocking α4* nAChRs in Leu9'Ala mice activates the indirect motor pathway resulting in a motor deficit. We also determined that α4* nAChRs involved in motor behaviors did not contain the α6 subunit, a nAChR subunit highly expressed in DAergic neurons suggesting that different nAChR subtypes modulating striatal DA release have separate functions in motor output. Conditioned place aversion and hypolocomotion, behaviors elicited during nicotine withdrawal, were also induced by DHβE in nicotine-naïve Leu9'Ala but not WT mice. Together these data suggest that DHβE globally reduces DA release in the CNS. In a separate project, I determined that α4* and α6* nAChRs modulate drug-induced hypnosis. Activation of nAChRs increased sensitivity to ketamine-induced hypnosis; whereas antagonizing nAChRs had the opposite effect. Additionally, α4 knockout (KO) mice were less sensitive to the hypnotic effects of ketamine, but α6 KO were more sensitive. High doses of ethanol induce an anesthesia-like state characterized by immobility, analgesia, and hypnosis. Testing the effects of ethanol hypnosis in α4 KO revealed that α4* nAChR do not play a large role in the acute effects of ethanol-induced hypnosis, but are involved in tolerance to this ethanol-induced behavior. The mechanisms of anesthetic-induced hypnosis are still largely unclear, despite the wide use of anesthesia. Future work on these receptors and their involvement in the anesthetic response will help to define a mechanism for hypnosis and improve the use of anesthetic drugs.

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5-HT	5-hydroxytryptamine (serotonin)
α4*	alpha 4 subunit containing
α-CtxMII	alpha conotoxin MII
ACh	acetylcholine
AMPH	amphetamine
ANOVA	analysis of variance
BAC	bacterial artificial chromosomes
BEC	blood ethanol concentration
CFC	contextual fear-conditioning
CNS	central nervous system
CPA	conditioned place aversion
D_1R	dopamine 1 receptor
D_2R	dopamine 2 receptor
DA	dopamine
DAergic	dopaminergic
DAPI	4',6-diamidino-2-phenylindole
DHβE	dihydro-beta-erythrodine
EEG	electroencephalography
EPM	elevated plus maze
ETIC	eticlopride
FSCV	fast-scan cyclic voltammetry
FSI	fast-spiking interneurons
fMRI	functional magnetic resonance imaging
GABA	γ-amino butyric acid
Glu	glutamate
GPe	globus pallidus external segment
GPi	globus pallidus internal segment
het	heterozygous
HEX	hexamethonium
HP	hot plate
ICSS	intracranial self-stimulation
i.p.	intraperitoneal
IPN	interpeduncular nucleus
KO	knock-out
L-dopa	L-3,4-dihydroxyphenylalanine
Leu9'Ala	leucine 9' alanine (mutation in α4 nAChR subunit)
L-dopa	levodopamine
LGIC	ligand-gated ion channel
LORR	loss of righting reflex
LDTg	lateral dorsal tegmentum
MEC	mecamylamine
MHb	medial habenula

MLA	methyllycaconitine
MSN	medium spiny neuron
NAc	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
NIC	nicotine
NMDA	N-methyl D-aspartate
PCP	phencyclidine
PD	Parkinson’s disease
PFC	prefrontal cortex
PLTI	persistent low–threshold spiking interneurons
PNS	peripheral nervous system
PPTg	peduncular pontine tegmental nucleus
REM	rapid eye movement
NREM	non-rapid eye movement
RBD	rapid eye movement sleep behavior disorder
SAL	saline
SEM	standard error of the mean
shRNA	short hairpin ribonucleic acid
SKF	SKF 82958, D_1R agonist
SN	substantia nigra
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
ST	striatum
TF	tail flick
TH	tyrosine hydroxylase
TM	transmembrane
TPP	tegmental pendunculopontine nucleus
VTA	ventral tegmental area
WT	wild-type
WTLM	wild-type littermates

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CHAPTER I.

INTRODUCTION

I.A. OVERVIEW

This thesis will examine nicotinic acetylcholine receptors and their role in motor function, withdrawal-like behavior, and the hypnotic effects of ketamine and alcohol. The beginning of the first chapter briefly outlines of the molecular structure and function of nicotinic acetylcholine receptors (nAChRs) in order to provide a basic understanding of these complex receptors. The second part of Chapter I focuses on dopamine in the motor pathway and the functions of nAChRs, specifically in the dorsal striatum. This is a preface for Chapter II, which investigates a motor deficit phenotype induced by blocking α4* nAChRs, using the nAChR antagonist DHβE, in a hypersensitive nAChR-expressing mouse. The third section is dedicated to nicotine withdrawal and provides a brief overview of the reward pathway, reviewing current literature on nAChR involvement in the nicotine withdrawal syndrome. This provides background on nicotine withdrawal in order link research in Chapter III investigating withdrawal-like behaviors induced by blocking α4* nAChRs in hypersensitive expressing nAChR expressing mice. The final section of Chapter I reviews anesthesia, specifically on ketamine, and the function of nAChRs in the anesthetic response, to supplement research presented in Chapter IV on nAChR involvement in the hypnotic effects of ketamine. Chapter V discusses the research presented in Chapters II-IV, and the implications and future directions of these studies.

I.B. Nicotinic Acetylcholine Receptors

STRUCTURE

Nicotine and the endogenous neurotransmitter acetylcholine (ACh) are potent agonists of excitatory ligand-gated cation channels known as nicotinic acetylcholine receptors (nAChRs) (Albuquerque et al., 2009). They are a part of the super-family of cys-loop ligand-gated ion channels (LGICs) that include γ-amino butyric acid (GABA), glycine and 5-hydroxytryptamine (5-HT) receptors (Gotti and Clementi, 2004, Millar and Gotti, 2009). All LGICs share a conserved sequence separated by linked cysteines on the amino (N) terminus (Zouridakis et al., 2009).

To date there are 17 known nAChR subunits (α1-α10, β1-4, γ, δ, and ε) (Albuquerque et al., 2009). Muscle nAChRs, which mediate the fast transmission at the skeletal neuromuscular junction, are pentameric and formed by the co-assembly of α1, β1, γ, δ, and ε subunits (Gotti et al., 2009, Zouridakis et al., 2009). In embryonic stages, muscle nAChRs express $\alpha1_2$, β1, γ, and δ, but in adults the γ subunit is replaced by the ε subunit. In these receptors, the ligand binding domain rests at the interface between α and γ, δ, and ε subunits (Zouridakis et al., 2009).

Neuronal nAChR are expressed throughout the nervous system. Twelve neuronal nicotinic acetylcholine receptor (nAChR) subunits have been identified as α subunits (α2-α10) and β subunits (β2-β4). Neuronal nAChRs co-assemble as homomeric or heteromeric pentamers. Homomeric receptors are composed of 5 identical α subunits, only formed by α7, α9, and α10. Agonists to these receptors bind at the interface between neighboring α subunits. Heteromeric receptors are composed of a combination of at least 2 α and 2 β subunits. Agonists to heteromeric nAChRs bind at the interface of the α (α2, α3, α4, or α6) and β (β2 or β4) subunits. Subunits α5 and β3 are considered accessory subunits because they do not contribute to ligand binding (Zouridakis et al., 2009).

Each subunit has 4 transmembrane (TM) domains (designated TM1-4) separating an extracellular N and C terminus (Figure I-1A) (Gotti and Clementi, 2004). Subunits share high conservation, except in the intracellular loop between the TM3 and TM4 domains which can vary in size and amino acid sequence (Albuquerque et al., 2009). α subunits are characterized by a conserved cysteine pair located on the N terminus, necessary for ligand binding, in LGICs. The β subunit has a complementary site that also contributes to binding (Gotti and Clementi, 2004, Millar and Gotti, 2009).

High-resolution structural imaging from the *Torpedo* muscle nAChR has helped to determine the structure of nAChRs (Zouridakis et al., 2009). In a formed receptor, the subunits are arranged in order: α, γ, α, β, δ (Millar and Harkness, 2008). The TM2 domain of each subunit creates the hydrophilic pore of the receptor, domains TM1 and TM3 protect TM2 from the lipid bilayer, and the TM4 domain remains the most exposed to the lipid membrane (Albuquerque et al., 2009, Gotti et al., 2009). The pore of the channel is wide at the top of the receptor and funnels down to a constricted TM2 ring that provides ion selectivity (Albuquerque et al., 2009). The cysteine loop of the N-terminus wraps around the outside of the neighboring subunit and creates a binding pocket at the interface of these two subunits (Albuquerque et al., 2009).

NAChRs can exist in 3 different transitional states: resting/closed, open, and desensitized (Gotti and Clementi, 2004). Ligand binding will switch resting/closed receptors into an open conformation by a twisting action of the TM2 region to favor a more hydrophilic conformation and allow Na+, Ca^{2+}, and K^{+} to flow down their electrochemical gradients leading to depolarization of the neurons (Albuquerque et al., 2009, Gotti et al., 2009). In response to prolonged exposure to agonist receptors transition into a desensitized state, in which the ligand is bound, but receptors are closed and passage of ions through the pore is blocked.

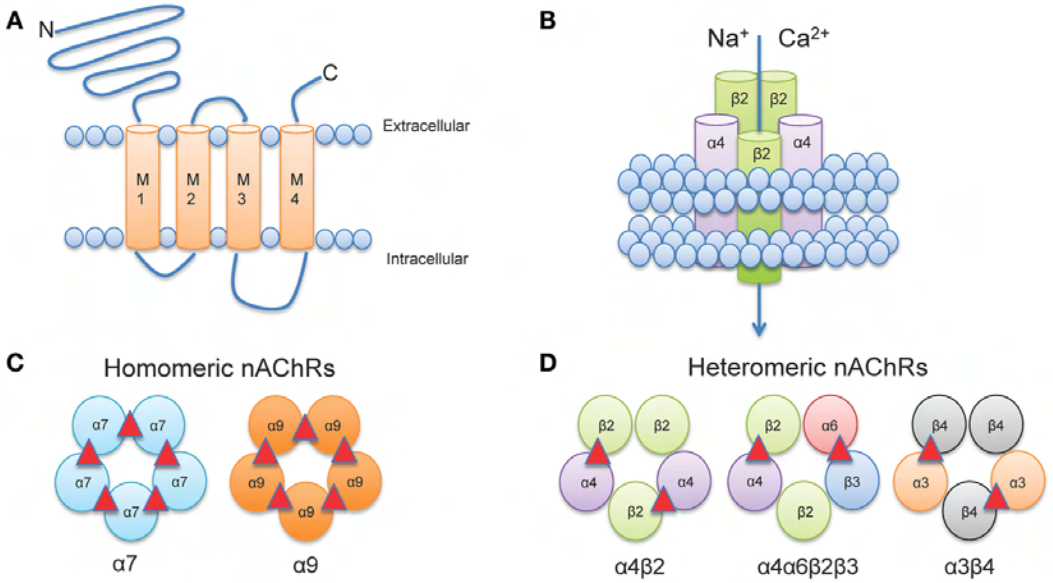

Figure I-1. Structure of nicotinic acetylcholine receptors
A) Schematic representation of an individual subunit. Each subunit contains an extracellular N and C terminus and 4 transmembrane domains (labeled M1-4). The variable loop is located intracellularly between M3 and M4 and is unique to each subunit. B) A pentameric nAChR embedded in a lipid membrane. C) and D) Examples of homomeric and heteromeric receptors. Red triangles indicate ACh-binding sites. Figure was taken with permission from (Hendrickson et al., 2013).

FUNCTION

Subunit composition determines the pharmacological and biophysical properties of nAChRs. Distinct nAChR subtypes also exhibit distinct expression patterns in the central nervous system (CNS). Homomeric α7 nAChRs are widely expressed throughout the brain. They are characterized by low affinity (in the µM range) to ACh, high Ca^{2+} permeability, a large conductance, rapid

desensitization, and high sensitivity (nM concentrations) to the nAChR antagonist α-bungarotoxin (Gotti and Clementi, 2004, Albuquerque et al., 2009). Rapid desensitization is thought to prevent excitotoxicity by abundance of Ca^{2+} influx (Gotti and Clementi, 2004). The densest population of α7 nAChRs is found in the hippocampus where they modulate glutamate and GABA release from pre-synaptic terminals, depolarize the membrane, and initiate intracellular processes in post-synaptic cells (Gotti and Clementi, 2004). α7 is normally found in a homomeric conformation, however heteromeric α7* nAChRs have been described in hippocampal interneurons and the intracardiac and superior cervical ganglia (Gotti and Clementi, 2004).

Heteromeric α4β2* nAChR subtypes account for up to 90% of high affinity nAChR receptors expressed in a wide variety of neuronal subtypes throughout the CNS (Gotti et al., 2009). These receptors are characterized by their high affinity for nicotine (in the nM-low mM range) and acetylcholine. They are also activated by the partial agonist cytisine, blocked by dihydro-β-erythrodine (DHβE), and insensitive to methyllcaconitine (MLA) and choline (Albuquerque et al., 2009). The stoichiometry of α and β subunits, in an α4β2 nAChR may also influence nAChR biophysical and pharmacological properties. For example, receptors in the $(\alpha4)_3(\beta2)_2$ conformation have a lower affinity for nicotine than $(\alpha4)_2(\beta2)_3$. The stoichiometry also confers differences in antagonist sensitivities and Ca^{2+} permeability (Gotti et al., 2009, Millar and Gotti, 2009).

High affinity α4β2* nAChRs also may co-assemble with other subunits such as α5, α6 and β3. Incorporation of the α5 subunit increases ion conductance, Ca^{2+} permeability, desensitization rate, and agonist affinity (Ramirez-Latorre et al., 1996, Gotti et al., 2009, Millar and Gotti, 2009). The α4α5β2 population makes up about 20% of the high affinity receptors in the brain (Brown et al., 2007). The α4α6β2β3 nAChRs are mainly found in dopaminergic (DAergic) terminals and exhibit increased sensitivity to agonist compared to α4(non-α6)β2* nAChRs (Salminen et al., 2007). These receptors have a more dominant role in ACh-mediated dopamine (DA) release in the nucleus accumbens (NAc) rather than the dorsal striatum which is dominated by α4α5β2* nAChRs (Exley et al., 2012).

Chronic nicotine exposure will lead to a general upregulation of nAChRs in the brain, but not equally amongst all nAChRs subtypes and brain regions (Gotti et al., 2009). Overall, there is a greater extent of upregulation of high affinity nAChRs, mainly α4β2* nAChRs (Gotti et al., 2009). Radioligand binding of [^{125}I]epabatidine in chronic nicotine treated mice reveals a 20-30% increase in high affinity nAChR binding in cortical areas, dorsal striatum (ST), NAc, ventral tegmental area (VTA) and superior colliculus (Even et al., 2008). However, an upregulation of α7 nAChR expression, using the a7 nAChR antagonist α-[^{125}I]bungarotoxin, was measured in the cortex and hippocampus after chronic nicotine in mice (Marks et al., 1985). However, not all nAChRs are upregulated.

For example, incorporation of α5 in α4β2* nAChR renders α4α5β2* nAChRs resistant to upregulation (Mao et al., 2008). There is even greater complexity among the α6* nAChR population. This is exemplified by an upregulation of α6(non-α4)β2* nAChRs and a downregulation of α4α6β2β3 nAChRs (Perez et al., 2008). Furthermore, incorporation of the β3 subunit in α6* nAChRs confers resistance to nicotine-induced downregulation (Perry et al., 2007).

GENETICALLY MODIFIED MICE

The diversity of biophysical and pharmacological properties of nAChRs suggest that the variety of nAChRs subtypes is not just a mechanism for redundancy but rather a reflection of specific functional roles of each receptor subtype. Currently knock-out (KO) models have been created for subunits α2-7, α9 and β2-4 (Drago et al., 2003, Gotti and Clementi, 2004, Lotfipour et al., 2013). Various knock-in models, introducing mutations in subunits resulting in hypersensitive receptors, are available for subunits α3, α4, α6, α7, and β2 (Tammimaki et al., 2011). To determine subunit contribution to behaviors evaluated in this thesis, I used a combination of targeting nAChRs by using nAChRs agonists and antagonists and mice with genetic modifications in the α4 and α6 subunits.

In this thesis I utilize Leu9′Ala mice, which provide the ability to test for sufficiency of α4* nAChRs. The gain of function mutation was first discovered in

chick α7 subunit by Revah et al. and since then mutations have been reproduced in the α3-α7, α9, and β2-β4 subunits (Revah et al., 1991, Drenan and Lester, 2012). The point mutation from a leucine to an alanine resided at the 9' position in the α-helical TM2 pore-forming region of the subunit. This point mutation affects the gating properties of this channel so that receptors are 50-fold more sensitive to nicotine and ACh than WT receptors (Miyazawa et al., 2003, Tapper et al., 2004).

The Leu9′Ala mouse line was created by the knock-in approach where homologous recombination in embryonic stem cells replaced a WT α4 subunit gene allele with the mutant allele (Tapper et al., 2004). The advantage of this mutation is that it provides the ability to selective activate α4* nAChRs with low doses of nicotine or ACh that will not result in a response from WT receptors. For example, a low dose of nicotine induced a hypothermic response and conditioned a place preference in Leu9′Ala but not WT mice (Tapper et al., 2004).

To test for the necessity of α4* nAChRs, α4 KO mice are utilized. These mice lack the gene encoding for the α4 subunit, *Chrna4.* More specifically, a 750 bp fragment was deleted from exon 5 of *Chrna4* (Ross et al., 2000). This fragment encodes the first hydrophobic transmembrane domain, TM1, through the second cytoplasmic loop, thus rendering α4 receptors non-functional. α4 KO mice are

viable, fertile, and are born in Mendelian proportions with no gross abnormalities (Ross et al., 2000). Quantitative autoradiography indicates that the α4 KO mice lack almost all high affinity binding sites, except in the superior colliculus, retroflexus, medial habenula (Hb) and interpenduncular nucleus (IPN), thus verifying a reduction in nAChRs containing the α4 subunit. Additionally, mRNA levels of other nAChR subunits do not differ between WT and α4 KO mice, suggesting that compensation has not occurred upon deletion of the α4 subunit in mutant mice. In a battery of behavioral tests in α4 KO mice, nicotine did not induce hypolocomotion, rearing or sniffing behaviors apparent in WT mice, thus supporting the lack of α4* nAChRs (Ross et al., 2000).

KO mice with a genetic deletion of the α6 subunit were used to test for the necessity of α6* nAChRs. Homologous recombination was used to create a 4kb deletion replaced with a neomyocin cassette in *Chrna6* (Champtiaux et al., 2002). The deletion removed exons 1 and 2, which encoded the ATG initiator codon, the signal peptide, and the N terminus of the subunit therefore preventing transcription of subunit. *In situ* hybridization confirmed the α6 subunit was not expressed and no compensatory changes in the mRNA levels of other subunits were detected. α6 KO mice were viable and born in Mendelian ratios, had normal growth rates, and did not exhibit major physical or neurological defects.

I.C. Motor Behavior

THE BASAL GANGLIA AND THE REGULATION OF MOVEMENT

One of the major functions of the basal ganglia system is to regulate voluntary movement.

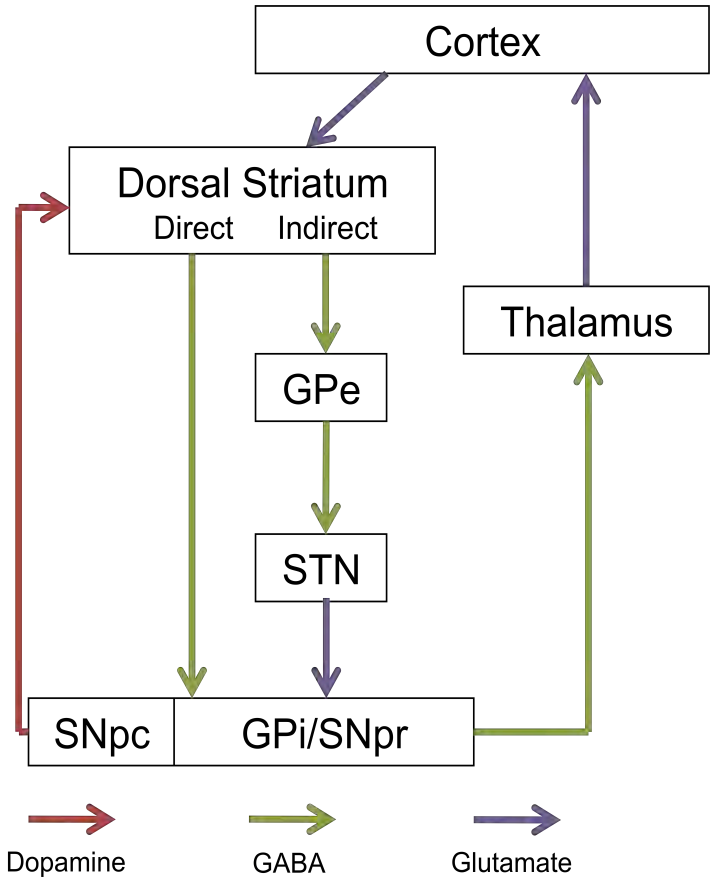

Figure I-2. The primary motor circuit in the CNS
This is a wire diagram illustrating the connectivity of the striatal circuit. There are two pathways, the direct and indirect pathways, which originate in the striatum. Activation of the direct pathway facilitates movement by activating the cortex and the indirect pathway inhibits the cortex to suppress movement.

Figure I-2 illustrates the simplified fundamental circuitry of the basal ganglia (Quik and Wonnacott, 2011). The substantia nigra pars compacta (SNpc) provides a DAergic input into the dorsal striatum, which is the driving force of the motor circuit. DA activates two populations of GABAergic neurons that regulate either the direct or indirect pathway, which ultimately inhibits or disinhibits the cortex. Movement is facilitated by activation of the direct pathway. The direct pathway begins in the striatum which sends GABAergic projections to inhibit the globus pallidus internal segment (GPi)/substantia nigra pars reticulata (SNpr). This results in a disinhibition of the thalamus, thus allowing activation of the the cortex and eventually facilitates movement. Suppression of movement is regulated by the indirect pathway, in which the GPi/SNpr is activated by subthalamic nucleus (STN) through the globus pallidus internal segment (GPe). Activation of the GPi/SNpr leads to an inhibition of the thalamus so cortex activation is blocked.

This separate and opposing regulation of the direct and indirect pathway has been the long-standing accepted model for movement output (Cerovic et al., 2013). Optogenetics have been used to demonstrate that direct pathway activation leads to increased movement (Kravitz et al., 2010). New evidence shows that this circuit may be more complex and both pathways work in concert with each other to facilitate movement, rather than the current model which

suggests there is a stronger activation of the direct pathways during movement and a stronger activation of the indirect pathway to stop movement. Cui et al. used an *in vivo* approach, combining fiber optics and time-correlated photon counting in the dorsal striatum, to show that the direct and indirect pathway were both activated during initiated movement (Cui et al., 2013). This implies that direct pathway is activated to facilitate wanted movement and simultaneous activation of the indirect pathway suppresses unwanted actions during movement.

THE STRIATUM

The direct and indirect pathways originate in the striatum, making this region central to the basal ganglia system (Figure I-3). Medium spiny neurons (MSNs) make up 90% of the neuronal population in the striatum and provide the GABAergic output of this brain region (Surmeier et al., 2010). These neurons regulate the indirect and direct pathways, which are modified by DA via excitatory or inhibitory G protein-coupled DA receptor expression. MSNs expressing excitatory D_1Rs (DA 1 receptors) activate the direct pathway, whereas D_2Rs (DA 2 receptors) are inhibitory and modulate striatal GABAergic output of the indirect pathway.

The indirect pathway is normally more active. Increases in DA will turn off the indirect pathway to inhibit suppression of movement and concurrently turn on the direct pathway to facilitate movement. D_1R KO mice had decreases in rearing during the open field test (Drago et al., 1994). D_2R KO mice exhibited akinesia, bradykinesia, abnormal posture, and abnormal gait (Baik et al., 1995, Kelly et al., 1998). Specific ablation of D_2Rs in the dorsal striatum, but not the ventral striatum, leads to hyperactivity in mice, indicating that there is an imbalance of the direct and indirect pathways (Kelly et al., 1998, Durieux et al., 2009). Naturally occurring dysfunction of these two pathways underlies movement disorders. For example, DAergic neurodegeneration in Parkinson's disease leads to indirect pathway overactivity resulting in difficulties in movement initiation, rigidity, freezing, and tremors.

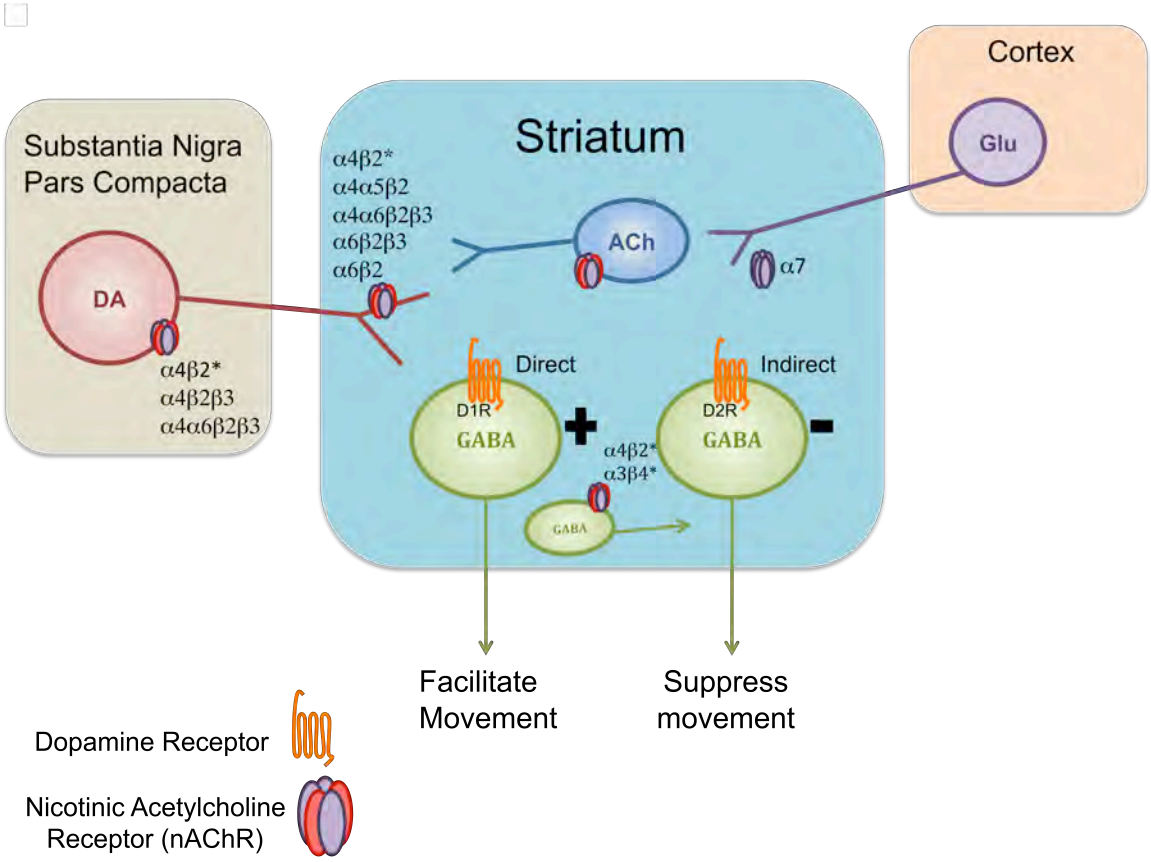

Figure I-3. The striatum
This is a schematic representation of the projections into and out of the striatum. The striatum receives a glutamatergic (Glu) input from the cortex and a DAergic input from the SNpc. GABAergic striatal output is mediated by these inputs as well as by cholinergic and GABAergic interneurons. GABAergic medium spiny neurons of the direct pathway contain excitatory dopamine receptors (D_1Rs) to activate the direct pathway and inhibitory dopamine receptors (D_2Rs) modulate the indirect pathway. Neuronal nAChRs are expressed throughout the striatum, except on MSNs, to modulate striatal output (Grady et al., 2007, McClure-Begley et al., 2009, Quik et al., 2009).

GABAergic and cholinergic interneurons make up about 5-10% of the striatal neuronal population and modulate striatal output. GABAergic interneurons are classified into fast-spiking interneurons (FSI) and persistent low–threshold spiking interneurons (PLTI) (Gittis and Kreitzer, 2012). FSIs receive strong

cortical innervations and regulate the majority of feedforward inhibition of both D_1R and D_2R MSNs, however FSIs preferentially project to D1R MSNs (Gittis et al., 2010, Gittis and Kreitzer, 2012). PLTIs may not have large consequences on striatal output because they make weak and sparse connections with MSNs, but they release neuropeptides to modulate the striatal circuitry (Gittis et al., 2010). Conversely, about 20% of PLTIs express neuropeptide Y, make strong connections with MSNs, and have a strong response to ACh, which suggests that these PLTIs play a larger role in striatal output (Gittis and Kreitzer, 2012, Luo et al., 2013).

Only making up about 1-2% of the striatal neuron population, tonically active cholinegeric interneurons bathe the striatum in the highest concentrations of ACh found in the brain (Kreitzer, 2009, Quik and Wonnacott, 2011). Anatomically, these large aspiny interneurons have extensive axons that project throughout the striatum and are innervated by cortical and thalamic projections (Calabresi et al., 2000). These neurons tonically fire action potentials and pause during synaptic DA input from the SNpc (Kreitzer, 2009, Quik and Wonnacott, 2011). Expression of D_2Rs on cholinergic interneurons also act as a feedback mechanism (Kreitzer, 2009).

Under resting conditions, a rhythmic pacemacker activity of SNpc DAergic neurons keeps continuous 10-20 nM concentration of DA in the striatum (Quik

and Wonnacott, 2011, Rice et al., 2011). Activation of SNpc DAergic neurons results in a bursting firing pattern, which increases striatal DA to concentrations in the high µM-low mM range. It also causes a pause in the tonic firing of cholinergic neurons to reduce ACh levels (Wonnacott, 2008, Kreitzer, 2009, Quik and Wonnacott, 2011). This activity shows that there is a fine balance of DA and ACh concentrations in the striatum.

Depleting ACh in striatal slices drops DA levels to as low as 10% of baseline DA concentrations (Zhou et al., 2001), suggesting that neuronal nAChRs are important mediators of DA release. Moreover, synchronous cholinergic stimulation can evoke terminal DA release, suggesting that terminally located nAChRs are sufficient to modulate DA release (Threlfell et al., 2012). There are a variety of nAChR subtypes involved in this response (see Fig. I-3) (Grady et al., 2007, McClure-Begley et al., 2009, Quik et al., 2009). Because of the heterogeneity of nAChR subtypes in DAergic nerve terminals, there are two major questions researchers are trying to answer: why is there redundancy of nAChRs in the striatum and are there separate functional roles for individual nAChR subtypes expressed in the striatum? Application of DHβE to striatal slices results in the same 90% reduction in DA concentrations as achieved by depleting striatal ACh, providing evidence that β2* nAChRs modulate ACh-evoked DA release (Zhou et al., 2001). Nicotine-stimulated DA release was also abolished in β2 KO mice, confirming that β2* nAChRs are necessary for nAChR

agonist evoked DA release (Grady et al., 2002). DA release studies in striatal synaptosomes revealed that the β2* nAChRs can be separated into α–Conotoxin MII (α–CtxMII) sensitive (α6*) and insensitive (α4*) nAChRs. The α–CtxMII sensitive nAChRs are composed of α4α6β2β3, α6β2β3, and α6β2 nAChRs and mediates about 30% of nicotine evoked DA release (Grady et al., 2002, Champtiaux et al., 2003, Cui et al., 2003, Salminen et al., 2007). The α–CtxMII insensitive nAChR population is composed of α4β2 and α4α5β2 nAChRs (Champtiaux et al., 2003, Salminen et al., 2004, Brown et al., 2007, Salminen et al., 2007, Grady et al., 2010). Of the α4* nAChR population, α4α5β2 and α4α6β2β3 nAChRs are only found on DAergic terminals, whereas α4β2 nAChRs are expressed on both DAergic terminals and GABAergic interneurons (Gotti and Clementi, 2004). Although α4α6β2β3* nAChRs are present in striatal DAergic terminals, α4α5* nAChR are more functionally dominant in the dorsal striatum (Champtiaux et al., 2003, McClure-Begley et al., 2009, Exley et al., 2012).

Evidence from fast-scan cyclic voltammetry (FSCV) reveal that control of DA release by α4β2* and α6β2* nAChRs is different. For example, increased DA release in α4 KO mice after burst firing (7-pulse) rather than a decrease observed in WT mice, suggest that α4* nAChR play a role in burst firing (Meyer et al., 2008). Alternately, α6β2* nAChR modulate ~65-80% of single-pulse nAChR mediated DA release (Perez et al., 2009, Perez et al., 2010). Together these data suggest that α6β2* nAChRs play a more prominent role tonic firing

(single pulse) important in DA maintenance, whereas α4β2* nAChR play a larger role in burst firing (repeated pulse), which is thought to control initiation and/or execution of movement (Exley et al., 2008, Meyer et al., 2008, Perez et al., 2008, Perez et al., 2009, Perez et al., 2010). However, the contribution of α4* and α6* nAChRs to movement still largely remains to be identified.

NAChRs AND MOVEMENT

To date very little is known about the role of nAChRs in motor output. One of the main challenges to identify their functional role in locomotor behavior is that pharmacological blockade of nAChRs has no effect on spontaneous locomotor activity in WT mice (Dwoskin et al., 2008, Jackson et al., 2009b). Increases in locomotor activity in the β2 and β3 KO mouse models are apparent in the open field test (Cui et al., 2003, Granon et al., 2003, Avale et al., 2008). Re-expressing the β2 subunit in the SNpc of β2 KO mice reverses this effect, suggesting the hyperactivity is a consequence of an imbalance of DA transmission (Avale et al., 2008). α4 and α6 nAChR subunit KO models do not reveal any major motor dysfunctions, most likely due to compensatory mechanisms (masking their true effects) (Ross et al., 2000, Champtiaux et al., 2002, Marubio et al., 2003). Since α4 and α6 KO mice have no gross locomotor deficits, they provide limited ability to identify functional roles of these nAChR subtypes in motor behavior.

Gain-of-function mouse models may provide more promising results. Basal hyperactivity measured in a gain-of-function α6* nAChR mice was reversed back to WT levels by knocking-out the α4 subunit (Drenan et al., 2010). This study demonstrates that that α4α6* nAChRs influence motor output. This suggests that nAChR subtypes expressed in the striatum have specific functions in motor behavior, which may be identified by combining pharmacology, KO and gain-of-function mouse models.

NAChRs AND PARKINSON'S DISEASE

Being second only to Alzheimer's disease, Parkinson's disease (PD) is the second most common neurodegenerative disease (Bosco et al., 2011). According to the National Parkinson's Foundation, it is estimated about 1% of the US population over 60 has PD and about 60,000 new cases are diagnosed a year. The resulting direct and indirect governmental costs are about 25 billion dollars per year (Bosco et al., 2011, Gazewood et al., 2013). PD is also the 14th leading cause of the death in the US causing about 20,000 deaths a year (Murphy et al., 2012).

PD is a disease of aging, with typical disease onset around the age of 60 (Gazewood et al., 2013). Primary clinical features of PD include bradykinesia,

rigidity, resting tremor, and postural instability (Schapira, 2009). PD patients also experience non-motor symptoms, such as autonomic deficits, psychiatric symptoms, behavioral changes, dementia, and sleep disorders, but treatment of PD is predominantly focused on alleviation of motor symptoms (Schapira, 2009, Quik et al., 2012, Gazewood et al., 2013).

Symptoms of PD are caused by neurodegeneration of DAergic neurons in the SNpc. Accompanying DAergic neuron loss, there is formation of cytosolic aggregates of proteins, mainly consisting of α synuclen and ubiquitin proteins, also known as Lewy bodies widespread throughout the brain (Bosco et al., 2011, Schapira and Jenner, 2011). The largest problem in PD diagnosis is that motor symptoms only become apparent after about 80% of DA neurons are lost. Furthermore, a true diagnosis can only be made post-mortem to identify the presence of Lewy bodies and loss of DAergic neurons in the SNpc.

In the first era of Parkinson's care, anti-cholinergic drugs were used to treat PD symptoms by correcting the imbalance of low DA and high acetylcholine levels in the brain (Brocks, 1999). The side effects of these drugs outweighed their effectiveness and so they were phased out after the discovery of dopamine replacement therapies such as dihydroxyphenylalanine (L-dopa) (Brocks, 1999, Gazewood et al., 2013). Despite their effectiveness, tolerance to L-dopa treatment occurs after long-term use. Furthermore, overuse of these drugs leads

to dyskinesias (involuntary movements) which can be as debilitating as PD symptoms (Quik et al., 2011a). Dopamine replacement drugs only manage motor symptoms, but do not treat non-motor symptoms nor delay or stop progression of disease. Current therapies are ineffective for long-term treatment, do not address non-motor symptoms, or target neurodegeneration, therefore there is a great need to identify novel targets for the development of therapeutic drugs that will address these issues.

Smokers have a lower incidence of PD (Sugita et al., 2001). Studies have revealed correlations between lowered PD risk and duration of smoking (Thacker et al., 2007, Chen et al., 2010). Current smokers had a 79% decreased risk of PD compared with non-smokers, but former smokers' risk was only decreased by 22% (Thacker et al., 2007). There is also an effect of recency of smoking, such that smoking cessation within 0-9 years provided reduced risk that persisted up to 24 years, but no neuroprotective effects were measured for smokers who had quit more than 25 years previously (Thacker et al., 2007). It has even been reported that second-hand smoke may provide protection against neurodegeneration in PD (Searles Nielsen et al., 2012).

Nicotine is most likely the agent in cigarettes that provides the neuroprotective effects. Protective effects of chronic nicotine treatment against drug-induced lesioning have been identified in neuronal cell lines, primary cell culture, and *in*

vitro studies of rodents and non-human primates (Quik et al., 2009, Takeuchi et al., 2009, Quik et al., 2012). Huang et al. showed that nicotine only provided protection from lesion-induced degeneration when administered before lesioning in rats and monkeys, but not after (Huang et al., 2009). Furthermore, nAChR subunits are differentially affected by lesioning and nicotine neuroprotection (Ryan et al., 2001). Parkinson's patients and lesioned monkeys and rodents exhibit preferential declines in expression of $\alpha4\alpha6\beta2\beta3$ nAChRs followed by declines in $\alpha6\beta2$* nAChRs in more severe lesions, while $\alpha4$(non-$\alpha6$)$\beta2$* nAChR are less affected (Bordia et al., 2007, Khwaja et al., 2007, Quik et al., 2011b). It is possible that the lack of $\alpha4\beta2$* nAChR loss may be a reflection of their expression on non-DAergic neurons that do not undergo degeneration. However, nicotine is not neuroprotective in $\alpha4$ KO mice, suggesting that $\alpha4$* nAChRs are necessary for nicotine neuroprotection against lesioning (Ryan et al., 2001). Further studies also show that in non-nicotine treated mice the $\alpha4\alpha6$* nAChRs are preferentially lost during nigrostriatal damage, however, nicotine can protect $\alpha4\alpha6$* nAChRs from this decline following nigrostriatal damage (Huang et al., 2009). Together this suggests that both $\alpha4$* and $\alpha6$* nAChRs may be good therapeutic targets for Parkinson's disease.

Drug trials using nicotine to treat PD symptoms have been inconclusive (Quik et al., 2011a, Quik et al., 2012). Out of 10 trials conducted, only 5 trials provided promising results for nicotine as a therapy and one study even showed negative

effects of nicotine on motor symptoms (Ebersbach et al., 1999, Quik et al., 2011a, Quik et al., 2012). It is possible that differences in nicotine administration and in dosing may account for these disparities.

There is still potential for nicotine as a neuroprotective agent, but this still remains untested in human trials. Studies in rodents and non-human primates, suggest that nicotine alleviates L-dopa-induced dyskinesias (Quik et al., 2007, Bordia et al., 2008). This implies that nicotine may also still provide alleviation from dyskinesias in humans (Quik et al., 2007). Lesion studies in rodents also suggest that the use of selective drugs targeting α4* and α6* nAChRs may provide better outcomes of neuroprotection and treatment of motor symptoms (Bordia et al., 2007, Khwaja et al., 2007, Huang et al., 2009, Quik et al., 2011a, Quik and Wonnacott, 2011).

I.D. Nicotine Withdrawal

According to the Centers for Disease Control (CDC), tobacco-related diseases cause about 440,000 deaths and results in medical costs of about 96 billion dollars each year in the United States alone (2008). Therefore, there is great personal and global incentive to quit smoking. However, the nicotine withdrawal syndrome is an uncomfortable state, resulting from nicotine abstinence, and is thought to be the driving force of relapse (Shiffman, 1979, Kenny and Markou,

2001). Commonly experienced symptoms include gastrointestinal disturbances, nausea, tobacco cravings, tremors, cognitive deficits, irritability, drowsiness, depression, and anxiety (De Biasi and Salas, 2008). Intensity of the withdrawal syndrome and likeliness to remain abstinent are negatively correlated with duration of smoking (Hughes et al., 2008). Smoking cessation therapies are available, but only increase cessation success to 20% from 3-5% when nicotine cessation therapies are not used (Nides, 2008). Therefore, better therapeutic approaches need to be developed to aid in smoking cessation to increase success rates.

NICOTINE IN THE REWARD PATHWAY

Nicotine activates the reward circuitry, also known as the mesocorticolimbic pathway. In this pathway, DAergic neurons originating in the ventral tegmental area (VTA) project to the nucleus accumbens (NAc) and the prefrontal cortex (PFC) (Markou, 2008, Hendrickson et al., 2013). Output of the VTA is determined by glutamatergic inputs from the NAc and PFC, cholinergic tone provided by the tegmental pendunculopontine (TPP) nucleus, and inhibition of VTA DAergic neurons by VTA GABAergic interneurons (Fig. 1-4). Nicotinic acetylcholine receptors expressed throughout the reward pathway contribute to the output of the NAc.

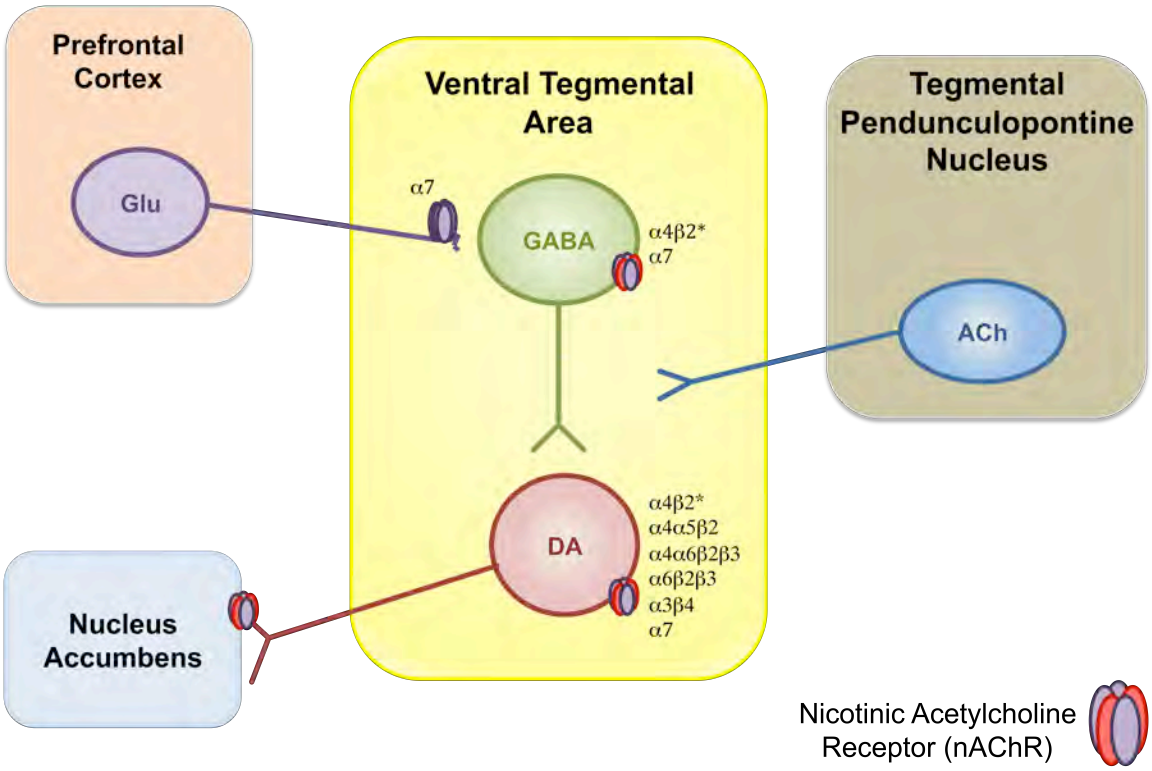

Figure I-4 nAChR expression in the mesocorticolimbic pathway
A simplified schematic of the reward circuitry displays nAChR expression (Hendrickson et al., 2013). The prefrontal cortex (green) and the tegmental pedunculopontine nucleus (TPP) send excitatory projections onto the VTA. Within the VTA, GABAergic (γ-Aminobutyric-acid, GABA) interneurons send inhibitory projections onto the DAergic neurons, which project to the NAc. After acute nicotine stimulation of the reward pathway, these complex interactions ultimately result in DA release into the NAc.

Nicotine, like all drugs of abuse, stimulates DA release in the NAc (Pietila and Ahtee, 2000, Laviolette and van der Kooy, 2004, Markou, 2008). Conversely, nicotine withdrawal is associated with decreased neuronal activity in the VTA leading to decreased extracellular DA levels within the NAc (Hildebrand et al., 1998, Liu and Jin, 2004). Decreased NAc DA levels also occur after systemic

administration of nAChR antagonists to precipitate withdrawal in nicotine dependent rats (Fung et al., 1996, Hildebrand et al., 1998, Gaddnas et al., 2002). Furthermore, local injection of nAChR antagonists into the VTA, but not the NAc, also precipitates a withdrawal phenotype in rats, implicating the VTA as a region of interest in nicotine dependence research (Hildebrand et al., 1999, Bruijnzeel and Markou, 2004).

Nicotinic acetylcholine receptors mediate the response to nicotine and ACh in the reward pathway. Their expression is highly diverse, indicated in Fig. I-4, and indicates selective nAChR subtype functions. Glutamatergic input is modulated by α7* nAChRs while DAergic output is modulated by α4* and α6* nAchRs. VTA GABAergic neurons also express α4* nAChRs therefore these receptors have a more complex modulation of VTA output.

CHRONIC NICOTINE UPREGULATES nAChRS

A withdrawal state cannot be induced without chronic drug treatment. One of the major neuroadaptations that occurs in response to chronic nicotine treatment is the upregulation of nAChRs in the reward circuit, which is thought to play a significant role in dependence and upon cessation of nicotine treatment (withdrawal). Increases in nAChR binding with radiolabelled nicotine have been measured in post-mortem human brains and in rodents exposed to chronic

nicotine (Govind et al., 2009). Chronic nicotine-induced upregulation measured in the midbrain of mice was reversed after 7 days of nicotine cessation (Marks et al., 1985, Pauly et al., 1996). Nicotine binding in the VTA also shows that greater receptor upregulation is associated with longer nicotine treatment (Pietila et al., 1998).

There is a greater upregulation of high affinity nAChRs compared with other nAChRs subtypes such as α7* nAChRs following chronic nicotine treatment (Marks et al., 1985, Nuutinen et al., 2005, Even et al., 2008). High affinity receptor upregulation is dependent upon β2 subunit expression. This was demonstrated by elimination of epabatidine binding in β2 KO mice after chronic nicotine exposure (McCallum et al., 2006). Specific up regulation α4β2 nAChRs was visualized, by attaching α4 and β2 subunits to yellow and cyan fluorescent protein respectively, in HEK293T cells and cultured midbrain neurons after chronic nicotine (Nashmi et al., 2003). Upregulation of the high affinity α4β2* nAChRs suggests these receptors are important for nicotine dependence.

Although increased nAChR binding occurs after chronic nicotine exposure, mRNA levels for α2, α3, α4, α5 and β2 subunits remained unchanged (Marks et al., 1992, Pauly et al., 1996). Peng et al. provided evidence, in both cultured cells and *Xenopus* oocytes, that upregulation of α4β2* nAChRs in response to chronic nicotine exposure is accompanied by a reduction in α4β2* nAChR

turnover rate and degradation, hence proposing this mechanism as a method for nAChR upregulation (Peng et al., 1994). Reviewed in Govind et al., other studies have also identified changes in cell surface turnover rates, receptor trafficking, subunit maturation and assembly, changes in receptor stoichiometry, inhibition of degradation, and nAChRs conformation changes as mechanisms to mediate posttranslational nAChR upregulation (Govind et al., 2009).

THE WITHDRAWAL SYNDROME

A nicotine withdrawal syndrome can be elicited in rodents after chronic nicotine administration (Malin et al., 1992, Isola et al., 1999, Kenny and Markou, 2001). Symptoms are categorized into somatic and affective symptoms. Somatic symptoms, also known as physical symptoms, include abnormal behaviors (e.g., jumping, teeth chattering, ptosis, tremors, shakes) and normal behaviors that occur more frequently during withdrawal (e.g., grooming, scratching, and rearing). Affective symptoms are mood disturbances such as anxiety, reduced locomotor activity, hyperalgesia, anhedonia (reward deficit), and aversion (De Biasi and Salas, 2008).

In rodents, the most common symptoms tested for an affective withdrawal syndrome are hyperalgesia (sensitivity to pain), anhedonia (decreased reward), anxiety, and aversion. The hot plate (HP) and tail flick (TF) tests are most

commonly used to assess hyperalgesia. To test if a drug is rewarding, intracranial self-stimulation (ICSS) is performed. Anxiety tests include elevated-plus maze, light-dark box test, and marble burying tests. Aversion is tested using classical Pavlovian conditioning to train mice to associate the aversive effects of a drug with a specific environment. Also, another measures for affective withdrawal that utilizes Pavlonian conditioning is contexual fear-conditioning (CFC).

Withdrawal research is currently focused on identifying the specific nAChR subtypes that modulate somatic or affective withdrawal symptoms. Phenotypes in rodents chronically treated with nicotine can be elicited by either discontinuation of nicotine administration (spontaneous withdrawal) or precipitated by administration of selective nAChRs antagonists. Therefore, a combination of pharmacology and nAChR subunit KO mouse models can be used to identify which subunits modulate the different aspects of nicotine withdrawal.

Initial studies of the nicotine withdrawal syndrome revealed that systemic administration of non-selective nAChR antagonists mecamylamine elicit both somatic and affective withdrawal symptoms (Malin et al., 1994, Suzuki et al., 1996, Watkins et al., 2000). Intraventricular infusion of hexamethonium (a non-selective antagonists that does not cross the blood brain barrier), as well as infusion of mecamylmaine into the VTA, IPN, and Hb could also induce somatic

withdrawal symptoms, showing a strong involvement of the CNS in nicotine withdrawal (Malin et al., 1997, Hildebrand et al., 1999, Salas et al., 2009). Identification of nAChRs subtypes involved in somatic and affective withdrawal symptoms are reviewed in Tables I-1 and I-2.

Table I-1. Neural nAChRs subunits modulating classical nicotine withdrawal in rodents identified by pharmacological nAChR targeting

Drug	Subunit	Somatic	Affective						Reference
			HP	TF	EPM	CPA	CFC	ICSS	
HEX (s.c. or i.p.)	ns	-							(Malin et al., 1997)
		-	-	-	-				(Damaj et al., 2003)
		+		-	-				(Grabus et al., 2004)
						+			(Jackson et al., 2009a)
HEX (i.c.v.)	ns	+							(Malin et al., 1997)
Chlorisondamine (s.c.)	ns	+							(Hildebrand et al., 1997)
		+						-	(Watkins et al., 2000)
Chlorisondamine (i.c.v.)	ns	+						+	(Watkins et al., 2000)
MEC (i.p. or s.c.)	ns	+							(Malin et al., 1994)
						+			(Suzuki et al., 1996)
		+							(Hildebrand et al., 1997)
		+				+		+	(Watkins et al., 2000)
		+	+	+	-				(Damaj et al., 2003)
		+		-	-				(Grabus et al., 2004)
		+	+		+	+			(Jackson et al., 2008)
								+	(Johnson et al., 2008)
								+	(Stoker et al., 2008)
								+	(Jackson et al., 2009a)
DHβE (i.p.)	α4β2*							+	(Epping-Jordan et al., 1998)
		-				+			(Watkins et al., 2000)
		+	-	-	+				(Damaj et al., 2003)
		-		-	-				(Grabus et al., 2004)
								+	(Kenny and Markou, 2005)
								+	(Stoker et al., 2008)
							+		(Portugal et al., 2008)
						+			(Jackson et al., 2009a)
α-CtxMII (i.c.v.)	α6*	-	-	-	-	+			(Jackson et al., 2009b)
MLA (i.p.)	α7*	+	+	-	-				(Damaj et al., 2003)
		-		-	-				(Grabus et al., 2004)
						-			(Jackson et al., 2009a)

CPA: conditioned place aversion, CFC: contextual fear conditioning, EPM: elevated plus maze, HP: hot plate test, i.c.v: intracerebroventricular infusion, i.p.: intraperitoneal injection, ICSS: intracranial self-stimulation, ns: non specific, s.c.: subcutaneous, and TF: tail flick test, +: positive for signs, -: negative for signs, blank: not tested

Table I-2. Neural nAChRs subunits modulating classical nicotine withdrawal in rodents identified by spontaneous or precipitated withdrawal in KO mice

KO mouse	Somatic	Affective						Reference
		HP	TF	EPM	CPA	CFC	ICSS	
α2 KO	-							(Salas et al., 2009) M
α3 KO								
α4 KO								
α5 KO	- -	+		+	+			(Jackson et al., 2008) M (Salas et al., 2009) M
α6 KO								
α7 KO	- - - +	- -	-			 +	 -	(Grabus et al., 2005b) S (Salas et al., 2007) M (Jackson et al., 2008) S/M (Portugal et al., 2008) S (Stoker et al., 2012) S/M
β2 KO	+ + +	 -		 -	 -	 +		(Salas et al., 2004) M (Besson et al., 2006) M (Jackson et al., 2008) S/M (Portugal et al., 2008) S
β3 KO								
β4 KO	- -	-	+				 +	(Salas et al., 2004) M (Stoker et al., 2012) S/M

Withdrawal was precipitated with mecamylamine (i.p.) or spontantous (S) in KO mouse studies. +: means signs were present (suggesting that the deleted subunit is not involved), -: negative for signs (suggesting the subunit is involved), blank: not tested, CPA: conditioned place aversion, CFC: contextual fear conditioning, EPM: elevated plus maze, HP: hot plate test, ICSS: intracranial self-stimulation, and TF: tail flick test

Observing and counting specific somatic behavioral events measures the somatic withdrawal syndrome. KO models have indicated α2*, α5*, and β4* nAChRs as modulating somatic signs. Precipitation of somatic, but not affective symptoms specifically CPA and anxiety, by α-Conotoxin AU1B implicates α3β4* nAChR in modulation of somatic symptoms only (Jackson et al., 2013). α7* nAChRs only partially modulate somatic withdrawal, indicated by only a slight reduction but not elimination of somatic signs in a KO model and precipitation of some somatic signs by MLA (Damaj et al., 2003, Grabus et al., 2005a, Salas et al., 2007). Although β2* nAChRs have been implicated by both DHβE and in a KO model for somatic withdrawal, apparent somatic behaviors were very limited, therefore it is understood that these receptors have a minor impact on somatic signs (Malin et al., 1998, Besson et al., 2006).

A variety of subunits modulate the affective withdrawal syndrome, however not all subtypes are involved in all affective symptoms (Tables I-1 and I-2). The β4 and α7 subunits modulate the reward deficits measured by increases in intracranial self-stimulation (ICSS) (Stoker et al., 2012). Additionally, α7* nAChRs are involved in hyperalgesic effects of withdrawal, but not in withdrawal induced CPA or anxiety (Damaj et al., 2003, Grabus et al., 2004, Grabus et al., 2005b, Salas et al., 2007, Jackson et al., 2008, Portugal et al., 2008, Jackson et al., 2009a, Stoker et al., 2012). Precipitation of anxiety and a CPA response by α-CtxMII in nicotine dependent mice has implicated α6* nAChRs in affective

withdrawal. Targeting α4β2* nAChRs with DHβE precipitates anxiety, cognitive dysfunction (CFC), CPA, and reduced reward. These affective withdrawal behaviors modulated by β2* nAChRs have been confirmed using β2 KO mouse (Jackson et al., 2008, Portugal et al., 2008). Furthermore, varenicline, a partial agonist of α4β2* and α6β2* nAChRs (and a current smoking cessation drug), alleviates CFC during nicotine withdrawal, thus further confirming their role and indicating β2* nAChRs as good therapeutic targets for nicotine cessation (Raybuck et al., 2008, Bordia et al., 2012). The only other receptor thus far implicated in an affective withdrawal syndrome are α6* nAChRs (Jackson et al., 2009b). Testing in α6 KO mice is required to confirm the function of α6* nAChRs in affective withdrawal symptoms.

BRAIN REGIONS ASSOCIATED WITH NICOTINE WITHDRAWAL

Providing the DAergic input to the NAc, it is no surprise that the VTA plays a central role in modulating nicotine withdrawal. Studies have identified this region as sufficient to induce a nicotine withdrawal syndrome. Hildebrand et al. showed that direct infusion of mecamylamine, a non-specific nAChR antagonist, into the VTA of nicotine-dependent mice decreased locomotor activity and elicited somatic withdrawal signs (Hildebrand et al., 1999). MLA infused into the VTA will also induce locomotor depression and lowered NAc DA levels, implicating α7* nAChRs (Nomikos et al., 1999). Interestingly, α4* nAChR upregulation by

chronic nicotine is selective to GABAergic and not DAergic neurons in the VTA (Nashmi et al., 2007). In a recent study, Tan et al. used optogenetic activation of GABAergic VTA neurons to induce a CPA in nicotine naïve mice (Tan et al., 2012). Together this suggests that aversion in nicotine withdrawal may be through a GABAergic mechanism mediated by $\alpha4\beta2$* nAChRs.

Functionally and anatomically connected to the mesocorticolimbic pathway, the habenula and interpenducular nucleus (IPN) make up an “anti-reward” circuit implicated by its response to aversive stimuli (De Biasi and Salas, 2008, Bianco and Wilson, 2009). Recently it has become of interest because it is sufficient to induce a somatic nicotine withdrawal syndrome. Optogenetic stimulation of GABAergic neurons in the IPN induces somatic withdrawal signs but not affective symptoms (as measured by anxiety) (Zhao-Shea et al., 2013). Somatic withdrawal signs can be precipitated by directly infusing mecamylamine into the habenula or IPN (Hughes et al., 2008, Salas et al., 2009). Together with KO data and high receptor expression, $\alpha2$*, $\alpha5$*, and $\beta4$* nAChRs are thought to modulate this somatic response (Salas et al., 2004, Hughes et al., 2008, Salas et al., 2009). Infusion of an antagonist selective for $\beta4$* nAChRs, SR16584, into the IPN also induced somatic withdrawal symptoms in nicotine dependent mice, implicating $\beta4$* nAChRs in the IPN are important for somatic withdrawal (Zhao-Shea et al., 2013). In the medial habenula and IPN, $\alpha3\beta4$* nAChRs can co-assemble with the $\alpha5$ subunit, however α-conotoxin AU1B precipitation of

somatic withdrawal signs in α5 KO mice suggest that modulation by α3β4* nAChRs is independent of α5 (Jackson et al., 2013).

In addition to somatic withdrawal, the medial habenula may also modulate affective withdrawal signs. Unpublished data from our lab shows that mecamylamine infusion into the medial habenula of mice precipitates anxiety in drug naïve mice (work done by Xueyan 'Alice' Pang). This may be mediated through α4* nAChRs based on functional α4* nAChR expression in the medial habenula and their known modulation of affective withdrawal (Epping-Jordan et al., 1998, Watkins et al., 2000, Damaj et al., 2003, Kenny and Markou, 2005, Portugal et al., 2008, Stoker et al., 2008, Fonck et al., 2009, Jackson et al., 2009a).

I.E. General Anesthesia

The first applications for inhalation drugs, such as chloroform and nitrous oxide, as a means to eliminate pain during surgery, were discovered in the 1840's (Rudolph and Antkowiak, 2004). Barbiturates were discovered about 60 years later and became the principle class of anesthetics for the next 50 years (Ferguson, 2010). These drugs were phased out in favor of "safer" drugs, like bromides and benzodiazepines, as a result of the addictive nature of barbiturates

that led to abuse and high rates of overdose. Since the discovery of anesthetics, much work has focused on creating a safe anesthetic state.

Today, general anesthesia is used in about 60,000 patients daily in the US alone, mainly for surgical procedures and in pre-and post-operative care (Brown et al., 2010). The common features of the anesthetic state induced by all anesthetic drugs are immobility, analgesia (pain relief), and hypnosis (sedation/loss of consciousness). Anesthetic drugs can be categorized into 3 groups (Table 1-3 modified from Solt and Forman) based on how well they induce each of these anesthetic properties (Eger et al., 1997, Solt and Forman, 2007, Brown et al., 2011).

Table I-3. Classification of General Anesthetic Drugs

	Group 1	**Group 2**	**Group 3**
General anesthetic drugs	Etomidate Propofol Pentobarbital	Nitrous Oxide Ketamine Xenon	Halogenated ethers (e.g. isoflurane and sevoflurane) Alkanes (e.g. halothane and chloroform)
Clinical Features	Strong hypnotics Weak immobilizers Weak analgesics	Weak hypnotics Weak immobilizers Potent analgesics	Strong hypnotics Strong immobilizers Weak analgesics
Molecular Targets	$GABA_A$ receptors	NMDA receptors AMPA receptors Neuronal nAChRs 2-pore K^+ channels	$GABA_A$ receptors NMDA receptors AMPA receptors Neuronal nAChRs 2-pore K^+ channels

Differences in potency for anesthetization and undesirable, potentially harmful side effects, such as respiratory and pulmonary effects, are also distinctive to each drug (Urban et al., 2006, Solt and Forman, 2007). Since drugs are not ideal in all anesthetic properties, cocktails of drugs are used to create an anesthetic state best fitting for the type of medical procedure, with the patient's medical history and current condition in mind.

Using more than one anesthetic drug can also reduce the concentration of each anesthetic required to induce the desired effect, which reduces the side effects of each drug (Urban et al., 2006). Although side effects may be reduced, they are not completely mitigated. Currently the mechanism of action of anesthetic drugs is unclear (see below), however it is clear that the anesthetic state is very complex and no one drug is likely to ever provide ideal anesthesia (Hemmings et al., 2005). With better understanding of the mechanism of these drugs, we may be able improve and enhance our current use of anesthetics.

MECHANISM OF GENERAL ANESTHETICS

Lipid membrane disruption was the original hypothesized mechanism of anesthetic action (Perry et al., 1999, Tassonyi et al., 2002, Franks, 2008). Since then, it has been discovered that anesthetics mainly target voltage- and ligand-gated ion channels, mainly GABA and glutamate receptors (see Table 1-3)

(Perry et al., 1999, Solt and Forman, 2007, Franks, 2008). Although individual anesthetics have specific molecular targets, they all induce hypnosis, analgesia, and immobility, albeit at varying degrees (Solt and Forman, 2007). Because of their similarities, it has been suggested that the anesthetic state uses the same pathways, despite differences in specific molecular targets of individual anesthetic drugs (Rudolph and Antkowiak, 2004).

It appears that anesthetics act through the sleep/wake circuits (Alkire et al., 2008, Allada, 2008, Franks and Zecharia, 2011). Distinct spontaneous encephalographic (EEG) activity under anesthesia, characterized by spindles (brief 7-14 Hz bursts of activity) and delta waves (1-4 Hz), is reminiscent of non-rapid eye movement (NREM) sleep (Alkire et al., 2008, Allada, 2008, Franks and Zecharia, 2011). Functional magnetic imaging (fMRI) and positron-emission tomography (PET) imaging during anesthesia largely reveal a reduction in whole brain activity, which also occurs during sleep (Alkire, 2008). During natural and anesthesia-induced sleep, greater depressions in brain activity are measured in the spinal cord, brainstem, cerebellum, midbrain, basal ganglia, superior frontal gyrus, posterior cingulate, basal forebrain, the insular cortex, prefrontal cortex, parietal and temporal association areas, occipitoparietal association cortices, and occipital cortex (Urban et al., 2006, Franks and Zecharia, 2011). Unfortunately, anesthetic drugs are not selective for specific brain regions, so it is unclear which regions are most important for mediating the anesthetic state.

The thalamus may be an important region involved in the effects of anesthetics because it is a key player in arousal. Reductions in blood flow and metabolism in the thalamus are measured during anesthesia-induced sleep (Alkire et al., 2008). Arousal was depressed when pentobarbital, a $GABA_A$ receptor agonist, was infused directly into the thalamus (Miller and Ferrendelli, 1990). On the other hand, arousal was induced by direct infusion of nicotine, a nAChR agonist, during severoflurane-induced anesthesia in rats (Alkire et al., 2007). These studies suggest the thalamus is important in modulating the duration of anesthesia-induced sleep through different molecular targets.

GENERAL ANESTHETICS AND nAChRS

Brain regions associated with anesthetic effects also received cholinergic signaling, suggesting that ACh and nAChRs modulate the anesthetic state (Fig. I-5) (Perry et al., 1999).

Halothane, enflurane, and ketamine decrease ACh turnover rates differentially throughout the brain, suggesting that modulation of the anesthetic response by ACh is diverse (Ngai et al., 1978). Additionally, the indirect cholinergic agonists, physostigmine and tacrine, are commonly used to advance emergence from anesthetics (Perry et al., 1999, Brown et al., 2010). Smoking can affect anesthesia, such that smokers are more resistant to the hypnotic effects of

propofol (Lysakowski et al., 2006). This is problematic because higher doses of propofol need to be administered to induce an anesthetic state in smokers, potentially leading to greater adverse side effects.

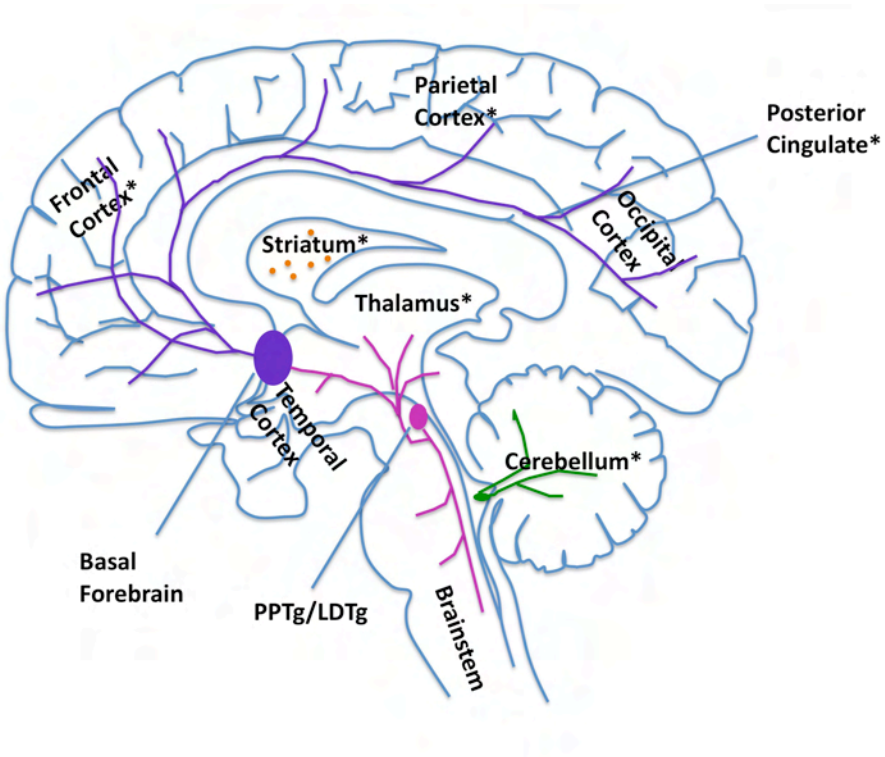

Figure I-5 Brain regions altered by anesthetics also receive cholinergic signaling.
A schematic of a human brain which highlights regions that are significantly changed by anesthetic drugs (Rudolph and Antkowiak, 2004, Alkire et al., 2008, Brown et al., 2010). Areas marked with an * are regions have been implicated in effects of an anesthetizing dose of ketamine in humans and rodents (Crosby et al., 1982, Davis et al., 1988, Hwang et al., 2012, Kim et al., 2012, Lee et al., 2013). The central cholingeric system in the brain has two main pathways originating in either the basal forebrain (purple) or the peduncular pontine tegmental nucleus (PPTg)-lateral dorsal tegmentum (LDTg) neurons (pink) (Perry et al., 1999, Scarr et al., 2013). Other main providers of acetylcholine in the brain are striatal cholinergic neurons (orange) and neurons in the vestibular nuclei that provide cholinergic signaling to the cerebellum (green).

Although not the primary target of anesthetics, *in vitro* studies show that nAChRs are inhibited by isolurane, halothane, sevoflurane, nitrous oxide, xenon, ketamine, thiopental, propofol, etomidate, and atacurium (Yamakura et al., 2000, Coates and Flood, 2001, Flood and Coates, 2002, Tassonyi et al., 2002). These *in vitro* systems have the advantage of identifying specific receptor subtypes that respond to anesthetics. For example, isoflurane and halothane inhibit $\alpha2\beta4$, $\alpha4\beta2$, and $\alpha7$ nAChRs while ketamine blocks $\alpha2\beta2$, $\alpha2\beta4$, $\alpha3\beta2$, $\alpha3\beta4$, $\alpha4\beta2$, $\alpha4\beta4$, and $\alpha7$ (Yamakura et al., 2000, Coates and Flood, 2001, Flood and Coates, 2002, Tassonyi et al., 2002). In many of these studies, clinically relevant doses of anesthetic could block nAChRs, however it is still uncertain if these subtypes influence the anesthetic state. Expression of nAChRs in *Xenopus* oocytes revealed that neuronal nAChRs are more sensitive to inhalational anesthetics than muscle nAChRs, suggesting that neuronal nAChRs mediated effects of general anesthetics (Violet et al., 1997).

Experiments in rodents have shown that nicotine treatment decreases enflurane, isoflurane, and sevoflurane analgesic effects and hypnosis duration (Flood et al., 2002b, Yan et al., 2009). Blocking nAChRs with mecamylamine or chlorisondamine had no effect on isoflurane-induced analgesia or hypnosis, even though nicotine modulated both these behaviors (Flood et al., 2002a, b). Mecamylamine also enhanced the analgesic effects of ketamine, but which

nAChR subunits are involved in this response is still unidentified (Udesky et al., 2005). Unfortunately, this is the extent of *in vivo* studies identifying the influence of nAChRs on anesthesia.

KETAMINE

Ketamine, an analog of phencyclidine (PCP), was developed to possess PCP's anesthetic properties without the negative hallucinogenic side effects (White and Ryan, 1996). Strong analgesic effects and lack of respiratory depression (negative side effects in most anesthetics) make ketamine the primary anesthetic for surgical procedures in the ill, elderly, and pediatric patients, pre- and post-operative care, and pain management (Alletag et al., 2012, Morgan and Curran, 2012, Prommer, 2012). It is also widely used in veterinary medicine (Morgan and Curran, 2012).

Ketamine is a unique anesthetic in that all other anesthetic drugs decrease brain activity, while ketamine has the opposite effect (Franks, 2008). Increased brain function may be the reason ketamine does not decrease respiration effects (which can be dangerous in the elderly, ill, or pediatric patients) giving ketamine an advantage over other anesthetic drugs (Brown et al., 2010). Instead of decreasing brain activity, ketamine acts by disrupting signaling (Alkire et al.,

2008, Lee et al., 2013). This aberrant activity may also be the cause of hallucinations and the mechanism for unconsciousness (Brown et al., 2010).

NMDA receptors are the primary molecular targets of ketamine, but *in vitro* studies have identified nAChRs as secondary targets. In PC12 cells, which express endogenous nAChRs, nicotine-evoked whole cell currents were inhibited by ketamine (Furuya et al., 1999, Sasaki et al., 2000). Expression of human nAChR subunits in *Xenopus* oocytes demonstrated ketamine inhibits $\alpha2\beta4$, $\alpha3\beta4$, $\alpha4\beta2$, $\alpha4\beta4$, $\alpha7$ nAChRs (Yamakura et al., 2000, Coates and Flood, 2001, Tassonyi et al., 2002). These studies further revealed that $\beta4$* nAChRs are more sensitive to ketamine than $\beta2$* nAChRs (Yamakura et al., 2000). There are discrepancies *in vitro* about the dose of ketamine required to block nAChRs; in some cases nAChRs were not inhibited by doses of ketamine that are considered clinically relevant to induce anesthesia (Furuya et al., 1999, Sasaki et al., 2000, Yamakura et al., 2000, Coates and Flood, 2001, Tassonyi et al., 2002). Therefore it is unclear if nAChRs contribute to the anesthetic effect of ketamine. In mice, analgesia effects of ketamine were enhanced by mecamylamine, however mecamylamine or DHβE did not influence the dose necessary to induced ketamine-induced hypnosis (Udesky et al., 2005). It still remains unclear if nAChRs play a role in the duration of hypnosis and immobility. With more knowledge about the contribution of nAChR subtypes to the anesthetic properties

of ketamine, nAChR drugs may be used to supplement ketamine to enhance its effectiveness and reduce negative side effects.

CHAPTER II.

A ROLE FOR α4(NON-α6)* NICOTINIC ACETYLCHOLINE RECEPTORS IN MOTOR BEHAVIOR

Contributions to Chapter II

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Author Contributions

Soll LG – genotyping, designed and performed experiments, performed data analysis, prepared figures, and wrote the manuscript for all published and unpublished material described in this dissertation.

Grady SR – provided α6 KO mice, designed, performed, and analyzed synaptosome data and co-wrote publication

Salminen O – designed, performed, and analyzed synaptosome data.

McIntosh JM – provided α-Conotoxin MII.

Tapper AR – designed experiments, performed data anaylsis, and co-wrote published manuscript.

Other Contributions

Guildford MJ, Ngolab J, and Pang X – genotyping

ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) containing either the α4 and/or α6 subunit are robustly expressed in dopaminergic nerve terminals in dorsal striatum where they are hypothesized to modulate dopamine (DA) release via acetylcholine (ACh) stimulation from cholinergic interneurons. However, pharmacological blockade of nAChRs or genetic deletion of individual nAChR subunits, including α4 and α6, in mice, yields little effect on motor behavior. Based on the putative role of nAChRs containing the α4 subunit in modulation of DA in dorsal striatum, we hypothesized that mice expressing a single point mutation in the α4 nAChR subunit, Leu9′Ala, that renders nAChRs hypersensitive to agonist, would exhibit exaggerated differences in motor behavior compared to WT mice. To gain insight into these differences, we challenged WT and Leu9′Ala mice with the α4β2 nAChR antagonist dihydro-β-erythroidine (DHβE). Interestingly, in Leu9′Ala mice, DHβE elicited a robust, reversible motor impairment characterized by hypolocomotion, akinesia, catalepsy, clasping, and tremor; whereas the antagonist had little effect in WT mice at all doses tested. Pre-injection of nicotine (0.1 mg/kg) blocked DHβE-induced motor impairment in Leu9′Ala mice confirming that the phenotype was mediated by antagonism of nAChRs. In addition, SKF 82958 (1 mg/kg) and amphetamine (5 mg/kg) prevented the motor phenotype. DHβE significantly activated more neurons within striatum and substantia nigra pars reticulata in

Leu9′Ala mice compared to WT animals, suggesting activation of the indirect motor pathway as the circuit underlying motor dysfunction. ACh evoked DA release from Leu9′Ala striatal synaptosomes revealed agonist hypersensitivity only at α4(non-α6)* nAChRs. Similarly, α6 nAChR subunit deletion in an α4 hypersensitive nAChR (Leu9′Ala/α6KO) background had little effect on the DHβE-induced phenotype, suggesting an α4(non-α6)* nAChR-dependent mechanism. Together, these data indicate that α4(non-α6)* nAChR have an impact on motor output and may be potential molecular targets for treatment of disorders associated with motor impairment.

II.A. Introduction

Balanced dopamine (DA) concentrations in striatum (ST) are essential for proper functioning of the basal ganglia circuitry and voluntary movement (Rice et al., 2011). Pathologically low DA concentrations, as caused by progressive neurodegeneration of substantia nigra pars compacta DAergic neurons in Parkinson's disease, leads to motor dysfunction, including akinesia, bradykinesia, resting tremor and catalepsy (Martin et al., 2011b). DA release in striatum (as well as other brain regions such as prefrontal cortex and hippocampus) is, in part, modulated by nicotinic acetylcholine receptors (nAChRs), ligand gated cation channels expressed on DAergic cell bodies and terminals, which are activated by the endogenous neurotransmitter, acetylcholine (ACh), as well as by the addictive component of tobacco smoke, nicotine (Grady et al., 2007, Albuquerque et al., 2009, Tang and Dani, 2009). Indeed, within striatum, high basal levels of ACh are achieved via tonic activity of striatal large aspiny cholinergic interneurons, suggesting activation of DAergic terminal nAChRs as key regulators of DA release (Zhou et al., 2001, Quik and McIntosh, 2006, Threlfell et al., 2012).

There are at least three major high affinity populations of nAChRs expressed in DAergic neurons in substantia nigra pars compacta: Those that contain the α4 subunit (α4* nAChR, "*" indicates other subunits coassemble with α4 within a

pentameric nAChR complex), those that contain the α6 subunit (α6* nAChR), and those that contain both subunits (α4α6* nAChR) (Salminen et al., 2004, Grady et al., 2007, Salminen et al., 2007, Gotti et al., 2010). While the majority of data indicating an involvement of nAChRs in modulating DA release from DAergic nerve terminals stems from studies of rodent synaptosomes and striatal slices (Zhou et al., 2001, Salminen et al., 2004, Zhang et al., 2009, Exley et al., 2012, Threlfell et al., 2012), pharmacological blockade of these receptors in mice have little impact on motor behavior (Dwoskin et al., 2008, Jackson et al., 2009b). In addition, mouse models that do not express the genes encoding either α4 or α6 nAChR subunits reveal few motor deficits, perhaps due to compensatory mechanisms (Ross et al., 2000, Champtiaux et al., 2002, Marubio et al., 2003). Thus, the precise impact of α4*, α6*, and α4α6* nAChRs on motor behavior is unclear.

While knock-out mice provide insight into the necessity of a targeted nAChR subunit, an alternative strategy is to study mouse models harboring "gain-of-function" mutations in a nAChR subunit (Lester et al., 2003, Drenan and Lester, 2012). To date, mice with a gain-of-function mutation in both α4 and α6 subunits have been generated (Tapper et al., 2004, Drenan et al., 2008a). BAC-transgenic mice expressing α6* nAChR with a point mutation that causes agonist hypersensitivity are hyperactive in both a novel environment and in the home cage (Drenan et al., 2008a). However, hyperactivity is abolished by crossing

these animals with α4 KO mice, indicating that increased motor activity is a result of α4α6* nAChRs that are hypersensitive to ACh (Drenan et al., 2010). To date, motor activity of α4 gain-of-function mice has not been studied in detail. Therefore, we were interested in elucidating a role for α4* nAChRs in basal ganglia related-movement behavior by analyzing motor behavior in knock-in mice that express α4 nAChR subunits with a point mutation (a leucine mutated to an alanine, the Leu9′Ala line) in the second transmembrane pore-forming region rendering functional receptors 50-fold more sensitive to agonists including ACh (Tapper et al., 2004, Fonck et al., 2005). We hypothesized that, if endogenous ACh stimulation of α4* nAChRs were important for DA-dependent motor behavior, then blockade of these receptors in Leu9′Ala mice would have exaggerated effects helping to uncover the role of these receptors on motor output.

II.B. Materials and Methods

Animals Male and female (8- to 14- week-old) Leu9′Ala knock-in mice, α6 KO mice and their wild-type (WT) littermates were used for all experiments. The genetic engineering of Leu9′Ala and α6 KO mice have been described previously (Champtiaux et al., 2002, Tapper et al., 2004). These mice have been backcrossed to the C57BL/6J background for at least 9 generations. Mice, bred at University of Massachusetts Medical School or the Institute for Behavioral

Genetics, University of Colorado, were housed four mice/ cage, received food and water *ad libitum* and kept on a standard 12-h light:12 hr dark cycle. All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council (National Research Council, 1996) or the guidelines for care and use of mice provided by National Institutes of Health, as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School or the Animal Care and Utilization Committee of the University of Colorado.

Drugs Nicotine hydrogen bitartrate, methyllycaconitine citrate salt hydrate**,** hexamethonium, D-amphetamine hemisulfate salt**,** Chloro-APB hydrobromide (SKF 82958), S-(-)-eticlopride hydrocloride, nomifensine, pargyline, atropine sulfate, bovine serum albumin (BSA) and diisopropyl fluorophosphate (DFP) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Dihydro-β-erythrodine hydrobromide (DHβE) was purchased from Tocris Bioscience Bristol, UK. N-2-(hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) and sodium salt were products of BDH Chemicals distributed by VWR (Denver, CO). [^{3}H]-dopamine ([^{3}H]-DA) (25-40 Ci/mmol) and Optiphase Supermix scintillation cocktail were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA). α-Conotoxin MII (α-CtxMII) was obtained from Dr. J. Michael McIntosh

(University of Utah). All drugs administered to mice were dissolved in 0.9% saline and administered via intraperitoneal (i.p.) injection at the indicated doses.

Motor Characterizations Drug naïve mice were placed into novel cages and allowed about 30 minutes to habituate to the cage. At time point 0 min, mice were tested for akinesia, catalepsy, clasping and tremor (described below). Immediately after baseline testing, mice were injected with saline or DHβE and characterizations were conducted for each mouse at the indicated time points over a 180 min period. In preliminary experiments, the effects of DHβE on motor phenotypes including locomotor activity, catalepsy, tremor and akinesia was analyzed, comparing genders in Leu9′Ala mice. Because the resulting analysis revealed no significant effect of gender (data not shown), data from male and female mice were combined.

Akinesia Every 30 min, mice were placed into an empty cage and held by the tail so hind limbs were hovering above the floor with forelimbs in contact with the floor of the cage. The number of forelimb steps forward was counted for 30 s. This was repeated and the two trials were averaged together.

Catalepsy The forelimbs of mice were placed on a raised bar 5 cm from the floor. Latency to remove both forelimbs off the bar was measured for up to 2 minutes. Catalepsy was measured every 60 min.

Clasping and Tremor Mice were tested for clasping and tremor by raising a mouse by the tail for 30 seconds and giving a score to depict the degree to which the hind limbs were spread apart or for severity of a body tremor. The scoring for clasping was as follows: 0= hind limbs spread wide apart (normal position), 1= hind limbs 25% closed, 2= hind limbs 50% closed, 3= hind limbs 75% closed with periods of hind limbs clasped, 4= hind limbs fully clasped for 10 seconds. The severity of a body tremor was scored: 0= no tremor, 1= isolated twitches, 2= non-continuous tremors, 3= consistent tremor.

Locomotor Activity For all experiments measuring locomotor activity, mice were given saline injections once a day for 3 days prior to the experiment to reduce differences in locomotor activity due to stress from the injection and handling. Additionally, on the day of the experiment, mice were habituated to the room for 1 hr to reduce differences in locomotor activity due to changes in environment unrelated to the novel cage. To measure locomotor activity, mice were placed into an individual cage within an infrared photobeam frame (San Diego Instruments) and allowed to roam freely for 30 min. Locomotor activity was measured by quantifying the number of beam breaks. Mice were challenged with saline or DHβE and placed into the locomotor chamber at the times indicated. Locomotor experiments were counterbalanced such that mice were exposed to either saline or drug and then one week later, drug treatments were switched.

Thus, each mouse served as its own control. Additional drug treatments (MLA and nicotine) and blocking experiments were tested in separate groups of mice. On the day of the experiment, mice were pre-treated with saline, nicotine, SKF82958, eticlopride, or amphetamine followed by saline or DHβE 5 min later as indicated. For the amphetamine rescue experiment, mice were injected with DHβE followed by an injection of saline or amphetamine 15 min after the first injection. Mice were placed into locomotor chambers at the times indicated post injection and locomotor activity was measured for 30 minutes.

Immunofluorescence To avoid neuronal activation due to stress induced handling, all mice were injected with saline once a day for 3 days before the experiment. Separate groups of drug naïve Leu9′Ala and WT mice received either saline or DHβE and perfused 150 minutes later. Prior to perfusion, mice were deeply anesthetized with sodium pentobarbital (200 mg/kg i.p.) and then perfused transcardially with ice-cold 0.1 M phosphate buffer (PBS, pH 7.4) followed by 10 mls ice-cold 4% (W/V) paraformaldehyde (PFA) dissolved in 0.1 M PBS (pH 7.5). The brains were harvested and post-fixed in PFA solution for 4 hr and then cryoprotected in PBS containing 30% sucrose. Coronal sections (20 µm thick) containing the striatum (ST) (between 1.18 mm and 0.38 mm from bregma) and the substantia nigra (SN) (between -2.92 mm and -3.8 mm from bregma) were sliced on a microtome (Leica SM 2000 R, Leica Microsystems Inc., Bannockburn IL, USA) and collected into a 24-well plate containing 1x PBS.

Sections were washed for 5 minutes in 1x PBS, placed into 0.4% Triton X-100 in PBS (PBST) for 5 minutes, washed again with 1x PBS for 5 minutes, and then incubated in a blocking solution containing 2% BSA in PBS for 30 min. Sections were incubated with primary antibody for c-Fos (polyclonal, 1:400, Santa Cruz) and ChAT (monoclonal 1:100, Santa Cruz) or Tyrosine Hydroxylase (TH, monoclonal, 1:1000, Santa Cruz) in the blocking solution overnight at 4°C. Sections were washed three times for 5 minutes and incubated in blocking solution for 30 min followed by another incubation in the blocking solution containing secondary fluorescently-labeled antibodies, goat anti-rabbit Alexa Fluor® 488 and goat anti-mouse Alexa Fluor® 594 (1:800, Molecular Probes Inc., Eugene USA) at room temperature for 30 min. Sections were washed five times for 5 min/wash and then mounted on slides and covered using VECTASHIELD® Mounting medium (Vector laboratories, Inc., Burlingame, CA, USA). A fluorescence microscope (Zeiss, Carl Zeiss MicroImaging Inc, NY, USA) was used to identify c-Fos immunopositive neurons, by quantifying intensities that were at least two times higher than that of the average value of background (sections stained without primary antibody) using a computer-associated image analyzer (Axiovision Rel. 4.6). The TH stain was used to identify the location of the SNpc (DAergic) and the SNpr, which is non-DAergic and located below the SNpc.

[^{3}H]-Dopamine release The methods of Salminen et al, 2007 were followed. Briefly, freshly dissected striata were homogenized by hand in 0.5 ml ice-cold isotonic sucrose buffered with HEPES (5 mM, pH 7.4). After dilution to 2 ml, 0.5 ml aliquots were centrifuged at 12,000 x g for 20 min at 4^{o}C. Aliquots were stored on ice as pellets (maximum time 3 hours) until re-suspension in uptake buffer [128 mM NaCl, 2.4 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES (pH 7.5), 10 mM glucose, 10 µM pargyline, 1 mM ascorbic acid)] and incubated at 37^{o}C for 10 min. [^{3}H]-DA (0.1 µM) and DFP (10 µM) were added and incubation continued for 5 min longer. At this point 8 aliquots of the crude synaptosome suspension (80 µl each) were placed on filters on superfusion platforms and superfused with buffer (uptake buffer with 0.1% BSA, 1 µM atropine and 1 µM nomifensine added) at 22^{o}C for 10 min before stimulation with various concentrations of agonist or agonist + DHβE in buffer for 20s followed by buffer. Alternate aliquots were superfused with buffer alone for 7 min, followed by buffer containing α-CtxMII (50 nM) for 3 min to block activity of α6β2*-nAChRs and, subsequently, the same stimulation protocol. Fractions were collected (10s each) into 96-well plates for ~1 min before stimulation, during stimulation and ~ 3 min following stimulation using a Gilson FC204 fraction collector (Middletown, WI). After addition of Optiphase Supermix scintillation cocktail (0.15 ml/well) radioactivity was determined using a 1450 MicroBeta Trilux counter (Perkin Elmer Life Sciences-Wallac Oy, Turku, Finland). Data were

expressed as units of DA release [summed (cpm – baseline cpm)/(baseline cpm)] for fractions 10% or more above baseline.

Data analysis Behavioral immunohistochemical and DA release data were analyzed with t-test, one or two-way ANOVA with repeated measures as indicated. Post-hoc analysis was done using Bonferroni post-hoc tests. Data were analyzed using Graphpad Prism 5 software (Graphpad Software, La Jolla, CA, USA). DA release data were analyzed by curve-fitting using SigmaPlot 8.0 (Jandel Scientific, San Raphael, CA).

II.C. Results

Previous studies indicate that BAC transgenic mice expressing agonist hypersensitive α6* nAChRs are hyperactive and that hyperactivity is abolished with genetic deletion of α4 subunit expression (Drenan et al., 2010). To test the hypothesis that Leu9′Ala mice, which harbor a similar mutation in the α4 subunit rendering α4* nAChR hypersensitive to ACh and nicotine, are hyperactive, we measured baseline locomotor activity of WT and homozygous Leu9′Ala mice. Locomotor activity did not significantly differ between genotype (data not shown). To test the hypothesis that motor output in Leu9′Ala mice was more sensitive to endogenous ACh compared to WT, we challenged Leu9′Ala mice and their WT littermates with the competitive β2* nAChR antagonist, DHβE and motor

behaviors such as akinesia, catalepsy, clasping, tremor (Figure II-1) and locomotor activity (Figure II-2) were measured. Much like the DA 2 receptor (D_2R) agonist, quinpirole (Zhao-Shea et al., 2010), DHβE elicited a profound abnormal motor phenotype in Leu9′Ala mice compared to WT. Figure II-1, depicts WT and homozygous Leu9′Ala mice 90 min after an i.p. injection of saline or DHβE (3 mg/kg in WT and 1 mg/kg in Leu9′Ala, respectively). DHβE had little effect in WT mice (Fig. II-1a), but induced postural abnormalities, such as curvature of the back and tail and clasping of the limbs in Leu9′Ala mice (indicated by arrows, Fig. II-1b). Two-way repeated measures ANOVA of akinesia (Fig. II-1c) after DHβE in WT and Leu9′Ala mice revealed a significant effect of time ($F_{6,108} = 9.030$, $p<0.0001$), genotype ($F_{3,18} = 10.54$, $p<0.001$) and a significant interaction between genotype and time ($F_{6,108}= 9.966$, $p<0.0001$). Post-hoc analysis indicated DHβE significantly reduced number of forelimb steps forward in Leu9′Ala compared with WT mice at 30 min ($p<0.05$) and 60 min ($p<0.05$) post-injection. Comparison of the cataleptic effect of DHβE in WT and Leu9′Ala mice (Fig. II-1d), analyzed by a two-way repeated measures ANOVA, did not indicated a significant effect of genotype, time, nor an interaction. Analysis of clasping (Fig. II-1e), by two-way repeated measures ANOVA, indicated a significant effect of genotype ($F_{3,11} = 20.23$, $p<0.0001$), time ($F_{6,66} = 3.31$, $p<0.01$), and interaction between genotype and time ($F_{18,66} = 3.67$, $p<0.001$). Further post-hoc analysis revealed that DHβE induced significant increases in clasping in Leu9′Ala compared to WT mice at 60 ($p<0.05$), 90

($p<0.001$), 120 ($p<0.01$), 150 ($p<0.05$), and 180 ($p<0.01$) min. Two-way repeated measures ANOVA of DHβE-induced tremors (Fig. II- 1f) in Leu9′Ala and WT mice revealed a significant effect of genotype ($F_{3,15} = 4.983$, $p<0.05$) and interaction between genotype and time ($F_{18,90} = 2.437$, $p<0.05$). Post-hoc tests indicated a significant increase in tremor at 90 and 120 min post DHβE injection in Leu9′Ala mice compared to WT. There was no difference between WT and Leu9′Ala mice after a saline injection in any of the motor behavior assays.

To test the effects of blocking α4* nAChRs on locomotion (Figure II-2), saline (i.p.) or DHβE was administered to WT (3 mg/kg, i.p., Fig. II-2a, b) and Leu9′Ala mice (0.1, 0.3, 1 mg/kg, and 24 hr later (1 mg/kg), i.p., Fig. II-2c, d) and locomotor activity was recorded starting at 60 min after post-injection for 30 minutes. DHβE (3 mg/kg) did not significantly alter the time course of locomotor activity or total ambulation over 30 min in WT mice (Fig. II-2a, b) compared to saline injection. However, DHβE (1 mg/kg) significantly reduced Leu9′Ala locomotor activity compared to saline (Fig. II-2c, d). Two-way repeated measures ANOVA revealed a significant effect of drug treatment ($F_{3,11} = 4.637$, $p<0.05$), time ($F_{5,55} = 23.19$, $p<0.0001$) and a significant drug treatment × time interaction ($F_{15,55} = 3.578$, $p<0.001$). Interestingly, locomotor activity returned to baseline levels 24 hr after DHβE challenge indicating the effects of DHβE in Leu9′Ala mice were reversible. One-way ANOVA revealed a significant main effect of treatment in total ambulation in Leu9′Ala mice (Fig. II-2d) ($F_{3,11} = 3.646$,

$p<0.05$). Post-hoc analysis indicated that total locomotor activity after 1 mg/kg DHβE injection was significantly lower compared to saline ($p<0.01$) and 24 hr post DHβE challenge ($p<0.01$). Additionally, locomotor activity was unaffected by systemic administration of hexamethonium (1 mg/kg, i.p.), a nAChR antagonist which fails to cross the blood brain barrier, indicating that DHβE-induced motor dysfunction in Leu9′Ala is mediated by neuronal nAChRs (data not shown). MLA (10 mg/kg, i.p. Fig. II-2e and 2f), an α7 nAChR antagonist, had little effect on locomotor activity in Leu9′Ala mice compared to saline, indicating that the DHβE-induced hypolocomotion was likely a result of blocking α4β2* nAChRs. To test the hypothesis that DHβE is, in fact, acting as a competitive antagonist at α4β2* nAChRs, we injected Leu9′Ala mice with nicotine (0.1 mg/kg, i.p.) 5 min prior to challenge with DHβE (1 mg/kg, i.p.) and measured locomotor activity 1 hr post injection. Interestingly, nicotine (0.1 mg/kg, i.p.) prevented DHβE-induced hypolocomotion in Leu9′Ala mice (Fig. II-2e) indicating that the DHβE phenotype is specifically elicited by blockade of nAChRs.

DHβE induced motor deficits in Leu9′Ala mice through blockade of neuronal nAChRs via a CNS-specific mechanism, raising the possibility that DHβE is blocking α4* nAChRs in the basal ganglia. Importantly, α4* nAChRs are robustly expressed in DAergic neuron soma and terminals of the substantia nigra pars compacta where they modulate DA release in dorsal ST (Salminen et al., 2004). Thus, antagonizing these receptors could decrease DA release and elicit the

observed hypolocomotor phenotype. To test a potential involvement of DA, Leu9′Ala mice were pre-injected with SKF82958, a D_1R agonist (1 mg/kg, i.p), eticlopride, (1 mg/kg, i.p.), a D_2R antagonist, or the dopamine transporter competitive substrate, amphetamine (5 mg/kg, i.p.), 5 minutes prior to a DHβE challenge (1 mg/kg, i.p.) and locomotor activity was measured 90 min later (Fig. II-3a, b). Two-way repeated measures ANOVA revealed a significant effect of pre-injection ($F_{3,17}$ = 31.43, $p<0.0001$), time ($F_{5, 85}$ = 5.21, $p<0.001$) and significant interaction ($F_{15, 85}$ = 2.09, $p<0.05$). Post-hoc analysis indicated amphetamine and SKF82958 significantly increased locomotor activity compared to DHβE alone (Fig. II-3a). A one-way ANOVA of average total ambulation (Fig. II-3b) indicated a significant effect of drug pre-injection ($F_{3,14}$ = 25.77, $p<0.001$) and post-hoc analysis revealed that amphetamine ($p<0.001$) and SKF82958 ($p<0.05$) significantly increased locomotor activity compared to DHβE alone suggesting increasing DA release in striatum or activating D_1Rs is sufficient to prevent DHβE-induced hypolocomotion in Leu9′Ala mice. Although statistical analysis of eticlopride did not indicate a significant difference, there was a partial block of hypolocomotor activity indicated by increased locomotor activity counts above zero. In addition, all DA signaling compounds prevented rigidity (data not shown).

To test if increasing DAergic signaling could rescue DHβE-induced hypolocomotion in Leu9′Ala mice, we challenged Leu9′Ala mice with DHβE and

administered saline or amphetamine 15 min after the initial antagonist injection (Fig. II-3c, and 3d). Two-way repeated measure ANOVA revealed a significant effect of post-injection ($F_{1,6}$ = 19.91, $p < 0.01$) but not time. Post-hoc analysis indicated an amphetamine post-injection significantly increased activity compared to saline at each time point. Average total locomotor activity was also significantly increased after an amphetamine post-injection compared to saline ($t = 4.46$, $p < 0.01$). We also tested the action of SKF82958, eticlopride, or amphetamine alone in WT (Fig. II-4a and b) and Leu9′Ala (Fig. II-4e and f) mice in the absence of DHβE. A two-way repeated measures ANOVA of drug treatment over time in WT mice (Fig. II-4a) revealed a significant main effect of drug treatment ($F_{3,20}$ = 26.18, $p<0.0001$), time ($F_{5,100}$ = 16.25, $p<0.001$) and a significant interaction between drug treatment and time ($F_{15,100}$ = 3.42, $p<0.001$). Post-hoc analysis indicated a significant effect of amphetamine at various time points as indicated in FigII-4a. One-way ANOVA indicated that there was a significant effect of drug treatment ($F_{3,23}$ = 26.18, $p<0.0001$) on total locomotor activity summed over 30 min. Further post-hoc analysis revealed a significant increase in locomotor activity after amphetamine ($p<0.001$), a significant decrease after eticlopride ($p<0.05$), but no difference after SKF82958 compared to a saline injection. These drugs had similar effects in Leu9′Ala mice (Fig. 4c and d, a significant main effect of drug treatment, $F_{3,20}$ = 9.98, $p<0.001$ and time, $F_{5,100}$ = 6.94, $p<0.001$). There was also a significant effect of drug treatment when analyzing total locomotor activity over 30 min (Fig. II-4d) ($F_{3, 23}$ = 9.781,

p<0.001, One-Way ANOVA) and post-hoc analysis revealed amphetamine significantly increased locomotor activity (p<0.01), while eticlopride decreased locomotor activity (p < 0.05). Effects of DA signaling did not differ between genotypes (two-way ANOVA indicated a significant effect of drug treatment ($F_{3,40}$ = 22.66, p<0.0001) but not genotype nor a significant interaction). Taken together these data indicate that prevention of DHβE-induced hypolocomotion in Leu9′Ala mice was not an artifact of baseline hypersensitivity to DA signaling compounds in these animals.

Targeting DA receptor signaling through pharmacology was able to fully or partially alleviate DHβE-induced hypolocomotion in Leu9′Ala mice, suggesting a role for the direct and indirect basal ganglia pathways. To test this hypothesis, we used immunohistochemistry to examine c-Fos expression as a marker for neuronal activation in the dorsal ST (Fig. II-5a) and substantia nigra pars reticulata (SNr) (Fig. II-5b) of WT and Leu9′Ala mice in response to DHβE. For this experiment, WT and Leu9′Ala mice were challenged with saline (i.p.) or DHβE (1 mg/kg, i.p.) and the dorsal ST was immunolabled for c-Fos (a transcription factor and marker of neuronal activation (Cole et al., 1989) (red), ChAT (cholinergic acetyltransferase to identify cholinergic neurons, green) and the nucleic acid stain, 4',6-diamidino-2-phenylindole (DAPI) (blue). The SNr was stained for c-Fos (green), tyrosine hydroxylase (TH, a marker of catecholaminergic neurons, specifically of DAergic neurons in the SN pars

compacta, red), and DAPI (blue). Figures II-5a and b depict micrographs illustrating c-Fos expression after DHβE injection in Leu9′Ala mice. The total number of c-Fos immuno-positive neurons was counted in each brain region and analyzed by a two-way ANOVA. In the dorsal ST (Fig. II-5a), there was a significant effect of drug treatment ($F_{1,108}$ = 20.68, $p<0.001$), genotype ($F_{1,108}$ = 19.46, $p<0.001$), and a significant interaction between drug treatment and genotype ($F_{1,108}$ = 20.78, $p<0.001$). Post-hoc analysis indicated that there was a significant increase of c-Fos immuno-positive neurons in the dorsal ST in Leu9′Ala mice after DHβE ($p<0.001$) compared to WT. In the SNr there was a significant effect of drug treatment ($F_{1,52}$ = 31.44, $p<0.001$), genotype ($F_{1,52}$ = 30.88, $p<0.001$), and a significant interaction between drug treatment and genotype ($F_{1,52}$ = 31.44, $p<0.001$). Post-hoc analysis indicated that there was a significant increase of c-Fos immuno-positive neurons in the dorsal ST in Leu9′Ala mice after DHβE ($p<0.001$) compared to WT. c-Fos expression did not colocalize with ChAT immuno-positive (i.e. cholinergic) neurons in ST or TH immune-positive neurons in SNpc indicating that neurons activated by DHβE were likely GABAergic. DHβE did not significantly increase c-Fos expression in WT mice compared to saline in either brain region. Together these data indicate that there is activation of neurons within the motor pathway, specifically of the indirect pathway which controls/reduces movement in Leu9′Ala mice challenged with DHβE, indicated by activation of both dorsal ST and SNr (Gerfen and Surmeier, 2011).

To test the hypothesis that DA release in ST from Leu9′Ala mice is more sensitive to nicotinic agonists compared to WT, we compared ACh-evoked and nicotine-evoked DA release from ST synaptosomes of Leu9′Ala homozygous, heterozygous, and WT mice. Both ACh- and nicotine-stimulated DA release from striatal synaptosomes was concentration-dependent in all three genotypes (Fig. II-6). The total concentration-response relationship for ACh-mediated DA release in Leu9′Ala heterozygous and homozygous was only slightly changed compared to WT synaptosomes with a small shift to lower EC_{50} values (Fig. II-6a, Tables II-1 and II-2). A somewhat larger leftward shift was seen in Leu9′Ala heterozygous and homozygous synaptosomes compared to WT using nicotine as agonist (Fig. II-6d, Tables II-1 and II-2). To determine the relative contribution of α4* versus α6* nAChRs on agonist-evoked DA release, we measured release in the presence of α-conotoxin MII (α-CtxMII), an α6* nAChR selective antagonist (Fig. II-6b and 6e). The α-CtxMII-resistant fraction of DA release (mediated by $\alpha4\beta2$*-nAChR) was more sensitive to both ACh and nicotine in heterozygous and homozygous Leu9′Ala synaptosomes compared to those from WT mice (Fig. II-6b, 6e and Table II-1) and maximum release was significantly decreased (Figure II-6b, 6e and Table II-2). In contrast, the α-CtxMII-sensitive component of DA release (mediated by $\alpha6\beta2$*-nAChR) in Leu9′Ala homozygous synaptosomes was not significantly more sensitive to either agonist compared to heterozygous or WT synaptosomes (Fig. II-6c, 6f and Table II-1). However, R_{max} was

significantly increased for the α-CtxMII-sensitive component of nicotine stimulated DA release (Fig. II-6c, 6f and Table II-2). Together, these data indicate that α4(non-α6)* nAChRs in striatal synaptosomes are hypersensitive to agonist in Leu9′Ala heterozygous and homozygous mice.

As blockade of α4β2* nAChRs using DHβE elicited a hypolocomotor phenotype in Leu9′Ala mice, we next measured the inhibitory-response relationship for DHβE on agonist evoked DA release in Leu9′Ala and WT synaptosomes (Fig. II-7) as well as determined K_i values for each genotype for DHβE inhibition of ACh-evoked release (Table II-3). At α4(non-α6)*-nAChRs as well as at α6*-nAChRs, DHβE dose-dependently inhibited DA release evoked by either ACh (Fig. II-7) or nicotine (data not shown). Interestingly, evoked DA release at α4(non-α6)* nAChR in Leu9′Ala homozygous synaptosomes was significantly more resistant to DHβE than the WT or heterozygous Leu9'Ala under equivalent agonist activation (Fig. II-7 and Table II-3). This effect is largely caused by a decrease in EC_{50} value (~9-fold) at α4*-nAChR with some increase (~2-fold) in K_i value for DHβE (Table II-3). EC50 values for ACh at α6*-nAChR (Table II-1) as well as K_i values for DHβE at α6*-nAChR (Table II-3) were unchanged by the α4Leu9′Ala mutation despite the known existence of the α4α6β3β2-nAChR subtype in DAergic neurons of WT mice (Salminen et al., 2004, Salminen et al., 2007).

To determine the contribution of α6*-nAChRs to the DHβE-induced phenotype in Leu9′Ala mice, we crossed Leu9′Ala mice to α6 KO animals and measured locomotor responses after antagonist treatment (Fig. II-8). In α6 KO mice on an α4 WT background, DHβE did not significantly modulate activity compared to saline (Fig. II-8a, b). Interestingly, DHβE significantly reduced locomotor activity in Leu9′Ala homozygous mice on an α6 KO background compared to saline and resulted in a motor phenotype indistinguishable from Leu9′Ala mice on a WT background (Fig. II-8b, c).

II.D. Discussion

Hypersensitive α4 nAChRs do not affect basal levels of locomotor activity*

Previously**,** hyperactivity was reported in a BAC transgenic mouse line expressing hypersensitive α6* nAChRs, suggesting a functional role for this receptor subtype in baseline motor behavior (Drenan et al., 2008b). Hyperactivity in this mouse line was also dependent on expression of the α4 subunit as crossing the α6 hypersensitive line to an α4 KO mouse line abolished locomotor hyperactivity (Drenan et al., 2010). Interestingly, we observed no significant differences in baseline locomotor activity between Leu9′Ala and WT mice. Recently, Cohen et. al. reported hyperactivity in BAC α6* nAChRs was a consequence of α6* L9′S nAChR subunit copy number (Cohen et al., 2012).

Mice with lower copy numbers were not hyperactive compared to those with higher copy numbers. Since Leu9′Ala α4 mice were generated via homologous recombination and only express two copies of the Leu9′Ala α4 nAChR gene (Tapper et al., 2004), it is possible that hyperactivity would be observed in mice expressing additional copies of the mutant nAChR subunit gene. Alternatively, compensation may account for the lack of hyperactivity. Leu9′Ala mice developed with α4* nAChRs hypersensitive to ACh, therefore there may be compensatory nAChR receptor expression, replacement of nAChR subunits, or altered DA receptor expression. Indeed, α4* nAChR activity is down-regulated in homozygous Leu9′Ala thalamus and cortical synaptosomes compared to WT (Fonck et al., 2005). In addition, expression of α6* nAChRs is limited to DAergic nerve terminals (Drenan et al., 2008a); whereas α4* nAChRs are widely expressed in DAergic, GABAergic, and glutamatergic neurons within the nigrostriatal pathway (Champtiaux et al., 2003, Marubio et al., 2003, Wooltorton et al., 2003, Xiao et al., 2009). Lack of hyperactivity may be a consequence of α4* nAChR activity in non-DAergic neurons.

Antagonism of hypersensitive α4 nAChRs leads to motor deficits*

Antagonism of α4* nAChRs in Leu9′Ala mice evoked robust, reversible parkinsonian-like symptoms characterized by hypoactivity, akinesia, catalepsy, and tremor. These symptoms have been previously shown to be associated with

DA depletion. Therefore we hypothesize that the abnormal motor symptoms observed after blocking hypersensitive α4* nAChRs are an effect of low levels of DA in dorsal ST. Although absolute confirmation of our hypothesis requires measuring DA release in striatum *in vivo*, DHβE induced c-Fos expression in Leu9′Ala dorsal ST and SNpr suggests inactivation of the direct motor pathway and activation of the indirect motor pathway. Indeed, the motor phenotype was alleviated by 1) increasing DA release using amphetamine, 2) by increasing direct motor pathway activation directly through D_1R agonism, or 3) by antagonizing D_2Rs, thereby redirecting available DA to activate the direct pathway. DHβE had no measureable behavioral effect in WT mice potentially because blockade of WT α4* nAChRs may not reduce striatal DA concentrations sufficiently low enough to induce a measurable motor phenotype.

Effect of DHβE is independent of α6 subunit incorporation

Although α4* and α6* nAChRs are expressed in DAergic nerve terminals in striatum, their functional roles are not equal in this region. DA release in the ventral striatum, associated with reward based behaviors, is preferentially modulated by α6* and α4α6* nAChRs versus α4(non-α6)* nAChRs while the latter preferentially modulate DA release in the dorsal ST, associated with movement (Salminen et al., 2007, Drenan et al., 2008b, Exley et al., 2008, Exley et al., 2011, Exley et al., 2012). Approximately 25 to 30% of agonist evoked

[^{3}H]dopamine release from dorsal ST synaptosomes is mediated by α6* nAChRs resulting in a potentially more robust role for α4* nAChRs in modulating dorsal ST DA release (Salminen et al., 2004). In addition, approximately 50 to 60% of terminally expressed α6* nAChRs also contain an α4 subunit, which increases the receptors' sensitivity to ACh (Champtiaux et al., 2003, Marubio et al., 2003, Salminen et al., 2004, Salminen et al., 2007). Interestingly, the dose response relationship for agonist induced DA release in striatal synaptosomes was shifted to the left in Leu9′Ala mice compared to WT only for the α-CtxMII insensitive nAChR fraction indicating that α4(non-α6)* nAChRs were hypersensitive to agonist; whereas R_{max} was increased in the α-CtxMII sensitive fraction indicating a compensatory increase in α6* nAChRs in Leu9′Ala synaptosomes. Knocking out the α6 subunit in hypersensitive α4* nAChR Leu9′Ala mice had no effect on the motor phenotype induced by DHβE suggesting that observed behaviors are caused solely by antagonism of α4(non-α6)* nAChRs. It was recently reported that regulation of DA release probability in the dorsal ST is dominated by α4α5* nAChRs and that incorporation of α5 is critical for functioning of DA release from terminal expressed α4* nAChRs (Exley et al., 2012). Incorporation of the α5 subunit into α4* nAChRs increases agonist sensitivity and also increases permeability to Ca^{++} (Ramirez-Latorre et al., 1996, Tapia et al., 2007). In striatal synaptosome preparations, α4α5* nAChRs are responsible for ~70% DA release (Salminen et al., 2004). Additionally, these receptors are more resistant to desensitization (Grady et al., 2012). Thus it is likely that the DHβE-sensitive

nAChRs responsible for the abnormal motor phenotype in Leu9′Ala mice also contain the α5 subunit. These receptors may regulate basal DA concentrations in dorsal ST.

Implications of nAChRs mediated DA release

The relationship between cholinergic and DAergic signaling in the striatum is complex. Cholinergic neurons are tonically active and pauses in their activity coincide with changes from a phasic to bursting activity in DA neurons which increase DA release for neuronal signaling encoding messages for reward, learning, and motor behaviors (Rice et al., 2011). Depleting endogenous ACh decreases electrically evoked DA release by 90%, highlighting the balance of ACh and DA (Zhou et al., 2001). Additionally, synchronous cholinergic activation can increase DA release via terminal nAChRs; a process independent of neuronal activity (Threlfell et al., 2012). In WT mice, DHβE blocked this increase in DA release revealing that α4β2* nAChRs mediate this response. Our model suggests that α4(non-α6)* nAChRs play a significant role in maintaining DA levels for normal movement behavior which may be, in part, regulated by this uncoupled terminal DA release. While the DHβE-induced phenotype in Leu9′Ala mice observed in our study is likely exaggerated due to the agonist hypersensitivity of α4* nAChRs in this mouse line, it nevertheless indicates the importance of these receptors on motor function. Motor output in normal functioning basal ganglia may not be affected by antagonism of these receptors

most likely due to redundancy of DA modulatory mechanisms. However, under pathological conditions when a substantial number of DA neurons have degenerated, such as in Parkinson's disease, targeting these receptors may have a larger impact on motor deficits and may be ideal candidates for therapeutic drugs.

Much like the phenotype described here, we previously reported that Leu9′Ala mice treated with a DA D_2 receptor agonist, quinpirole, also develop reversible akinesia, rigidity, catalepsy, and tremor (Zhao-Shea et al., 2010). Here we show that directly blocking α4* nAChRs in Leu9′Ala leads to a more severe phenotype. More precisely, quinpirole-treated Leu9′Ala homozygous mice most closely resemble DHβE-treated Leu9′Ala heterozygous mice (data not shown); whereas hypolocomotion is more severe in DHβE-treated Leu9'Ala homozygous mice. Thus, it is possible that a functional interaction between D_2Rs and α4* nAChRs on DA terminals occurs such that activation of D_2Rs inhibits α4* nAChRs, potentially working in concert as a mechanism to regulate striatal DA levels (Quarta et al., 2006).

It is also possible these Leu9'Ala mice have altered connectivity of GABA interneurons that control activity of the direct and indirect pathways similar to that shown under low DA conditions, where 6-OHDA treatment and subsequent DA depletion led to an increase in connections between fast-spiking interneurons

and indirect pathway neurons (Gittis et al., 2011, Gittis and Kreitzer, 2012). Such a change could alter the importance of ACh levels controlled by DA D_2 receptors on cholinergic interneurons as well as activation of nAChRs on GABA interneurons. The result could be activation of indirect pathway neurons relative to the direct pathway upon decrease of ACh release or block of $\alpha4\beta2$ nAChR.

We have shown that antagonism of hypersensitive Leu9′Ala $\alpha4$* nAChRs in mice induces a robust, reversible motor phenotype, which can be prevented by targeting the DAergic system. $\alpha4$(non-$\alpha6$)* nAChRs play a major role in this response suggesting that these receptors may be critical for maintaining DA levels necessary for normal motor behavior. Together these data indicate $\alpha4$(non-$\alpha6$)* nAChRs in dorsal ST may represent therapeutic candidates for alleviating motor dysfunction, such as in Parkinson's Disease.

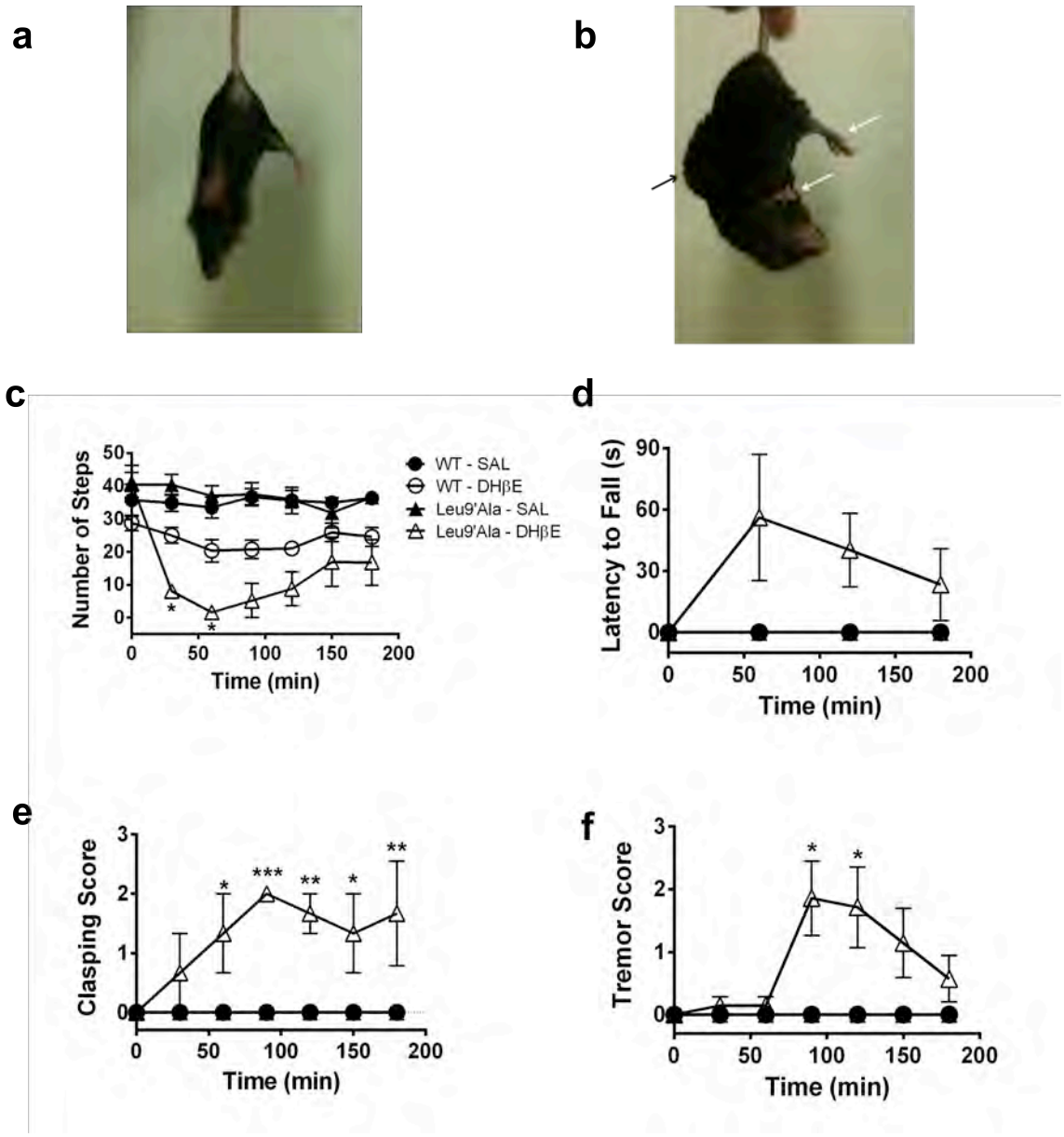
a
b
c
d
e
f
Number of Steps
Time (min)
WT - SAL
WT - DHβE
Leu9'Ala - SAL
Leu9'Ala - DHβE
Latency to Fall (s)
Clasping Score
Tremor Score

Figure II-1

Figure II-1. DHβE induces motor abnormalities in Leu9'Ala mice. a-b) Representative pictures of the phenotypic effect induced by DHβE 90 minutes after injection in a) WT (3 mg/kg DHβE, i.p.) and b) Leu9'Ala homozygous (1 mg/kg DHβE, i.p.) mice. Arrows highlight hind limb rigidity, arched back, and curled tail in Leu9'Ala mice. c-f) Motor symptoms were characterized every 30 min for 180 min in WT and Leu9'Ala mice immediately following an i.p. challenge of 3 or 1 mg/kg DHβE, respectively. c) Akinesia: Number of forelimb steps forward were counted for 30 seconds (WT SAL: n=4, WT DHβE: n=4, Leu9'Ala SAL: n=4, Leu9'Ala DHβE: n=7). d) Catalepsy: Latency for forelimbs to fall off a raised bar was recorded (WT SAL: n=4, WT DHβE: n=5, Leu9'Ala SAL: n=4, Leu9'Ala DHβE: n=6). e) Clasping of hind limbs during a 10 second period was measured: 0 = hind limbs spread wide apart, 1 = hind limbs are 25% closed, 2 = hind limbs are 50% closed, 3 = hind limbs are 75% closed with some clasping, 4 = constant clasping. (WT SAL: n=4, WT DHβE: n=5, Leu9'Ala SAL: n=4, Leu9'Ala DHβE: n=3). f) Tremor score: 0 = no tremor, 1 = isolated twitches, 2 = tremor with periods of calm, 3 = constant tremor (WT SAL: n=4, WT DHβE: n=4, Leu9'Ala SAL: n=4, Leu9'Ala DHβE: n=7). *p<0.05, **p<0.01, and ***p<0.001

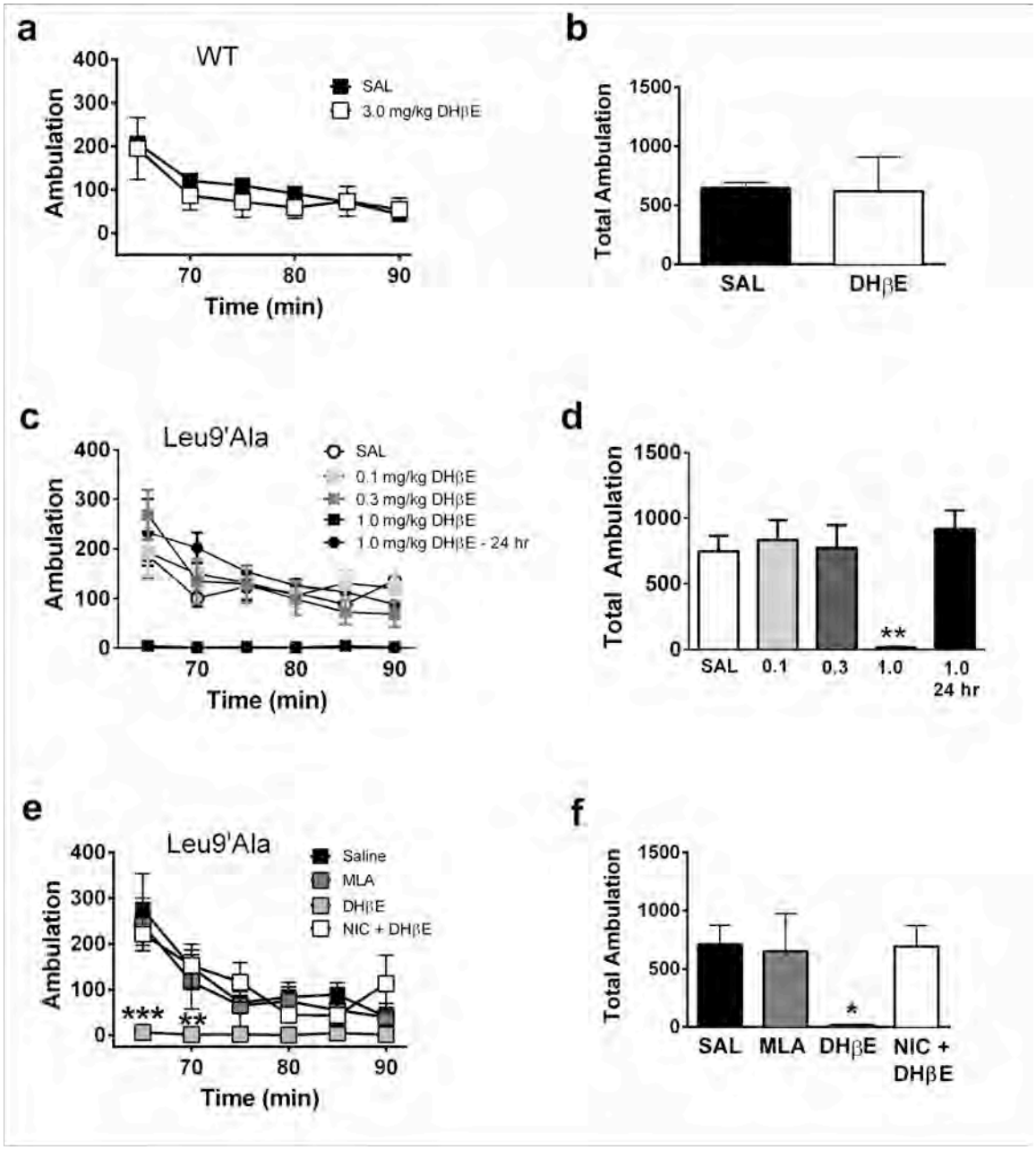
a
WT
SAL
3.0 mg/kg DHβE
Ambulation
400
300
200
100
0
70
80
90
Time (min)
b
Total Ambulation
1500
1000
500
0
SAL
DHβE
c
Leu9'Ala
SAL
0.1 mg/kg DHβE
0.3 mg/kg DHβE
1.0 mg/kg DHβE
1.0 mg/kg DHβE - 24 hr
Ambulation
Time (min)
d
Total Ambulation
**
SAL
0.1
0.3
1.0
1.0
24 hr
e
Leu9'Ala
Saline
MLA
DHβE
NIC + DHβE
***
**
Ambulation
Time (min)
f
Total Ambulation
*
SAL
MLA
DHβE
NIC +
DHβE

Figure II-2

Figure II-2. DHβE induces hypolocomotion in Leu9'Ala mice. a) WT mice were placed into novel cages 60 minutes after saline (i.p., n=4) or DHβE challenge (3 mg/kg, i.p, n=4) and locomotor activity was measured for 30 minutes. Each data point represents the 5 min sum of ambulatory activity. b) Bar graphs represent total ambulation during 30 min of locomotor activity in WT mice measured in panel a. c) Locomotor activity in homozygous Leu9'Ala mice 60 min after saline (i.p., n=4), 60 min after DHβE (0.1-1 mg/kg, i.p., n=4), and 24 hrs after DHβE (1 mg/kg, i.p, n=4). d) Total ambulation was quantified for each condition as in panel b. e) Saline (i.p., n=4), MLA (10 mg/kg, i.p., n=4), DHβE (1 mg/kg, i.p., n=4), and NIC (0.1 mg/kg, i.p.) 5 minutes before DHβE (1 mg/kg, i.p., n=4) was administered to Leu9'Ala mice and locomotor activity was measure 60 min later. f) Total ambulation over 30 minutes in Leu9'Ala mice was quantified for each condition in panel e and compared to saline control. *$p<0.05$ and **$p < 0.01$

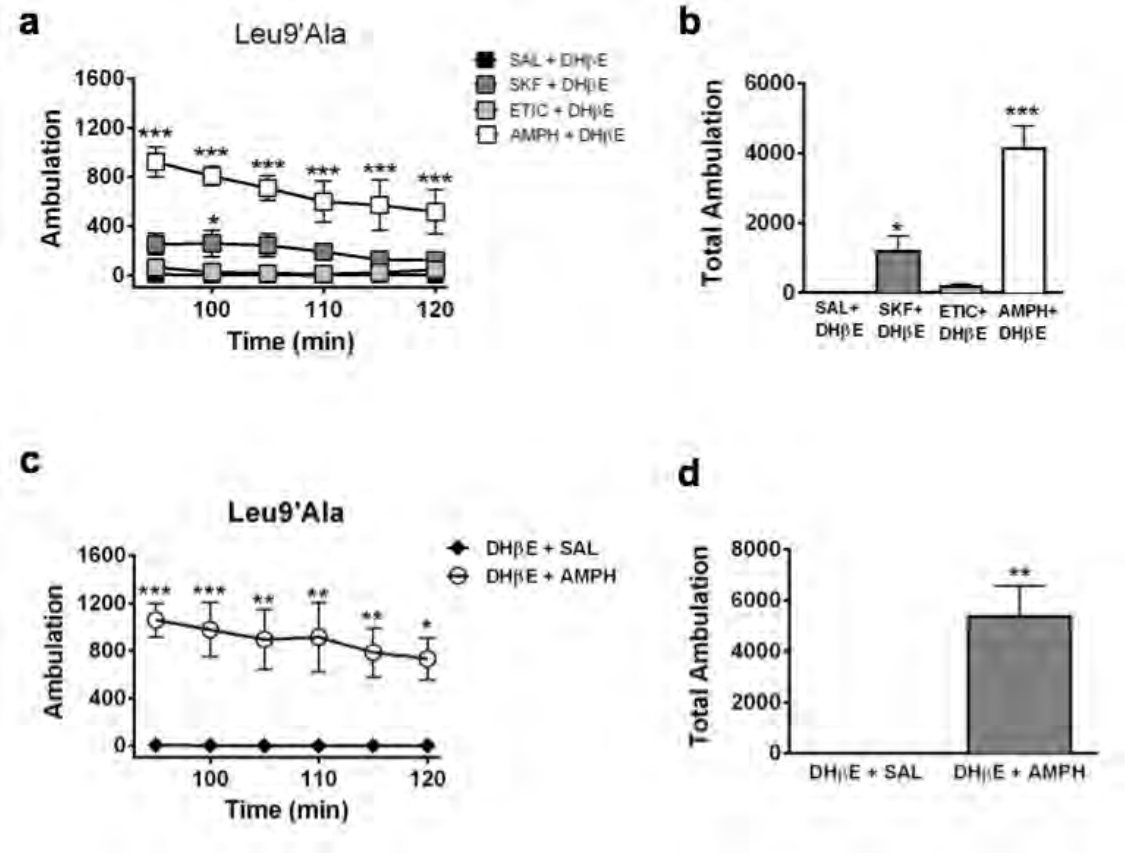

Figure II-3. Pharmacologically targeting DAergic signaling prevents DHβE-induced hypolocomotion in Leu9'Ala mice. a) Homozygous Leu9'Ala mice were placed into novel cages 90 min after administration of DHβE (1 mg/kg, i.p.) after preinjection of saline (SAL, 1 mg/kg; i.p.; n=4), SKF-38393 (SKF, 1 mg/kg, i.p., n=4), eticlopride (ETIC, 1 mg/kg, i.p., n=4), or amphetamine (AMPH, 5 mg/kg, i.p., n=4) and activity was measured for 30 min. Each data point represents the 5 min sum of ambulation at a given time point. b) Averaged 30 min sum of activity was quantified. c) Homozygous Leu9'Ala mice were challenged with DHβE (1 mg/kg, i.p.) followed by a saline (n = 4) or amphetamine (5 mg/kg, i.p., n = 4) injection 15 min later. Mice were placed into activity cages 90 min after the DHβE injection when motor deficits were at their peak. d) Averaged 30 min sum of activity was quantified. *p<0.05, **p < 0.01, ***p < 0.001.

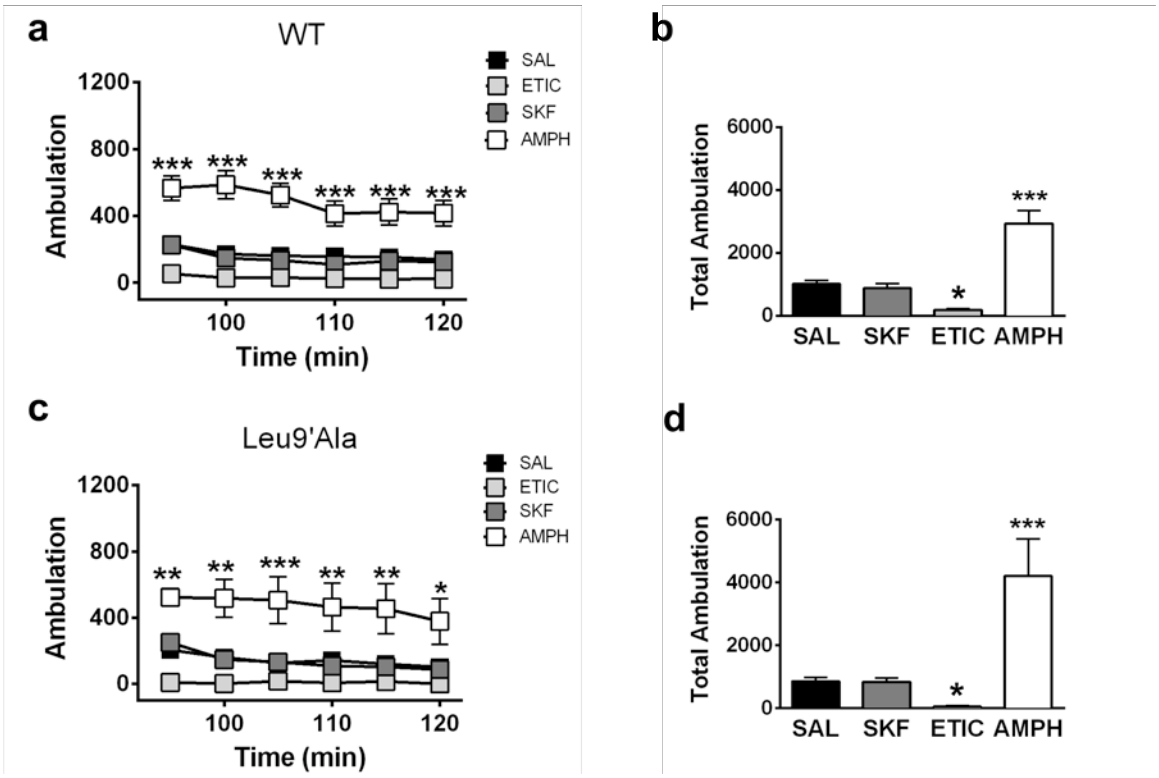

Figure II-4. DAergic signaling is not altered in Leu9'Ala mice compared to WT mice. Locomotor activity was measure 90 minutes after administration of saline (i.p., n=6), SKF (1 mg/kg, i.p., n=6), ETIC (1 mg/kg, i.p., n=6), and AMPH (5 mg/kg, i.p., n=6) in a) WT and c) Leu9'Ala. Total ambulation over 30 minutes for b) WT and d) Leu9'Ala mice after challenge of drugs from panel a and c, respectively as in Figure 3. *p<0.05 and ***p<0.001

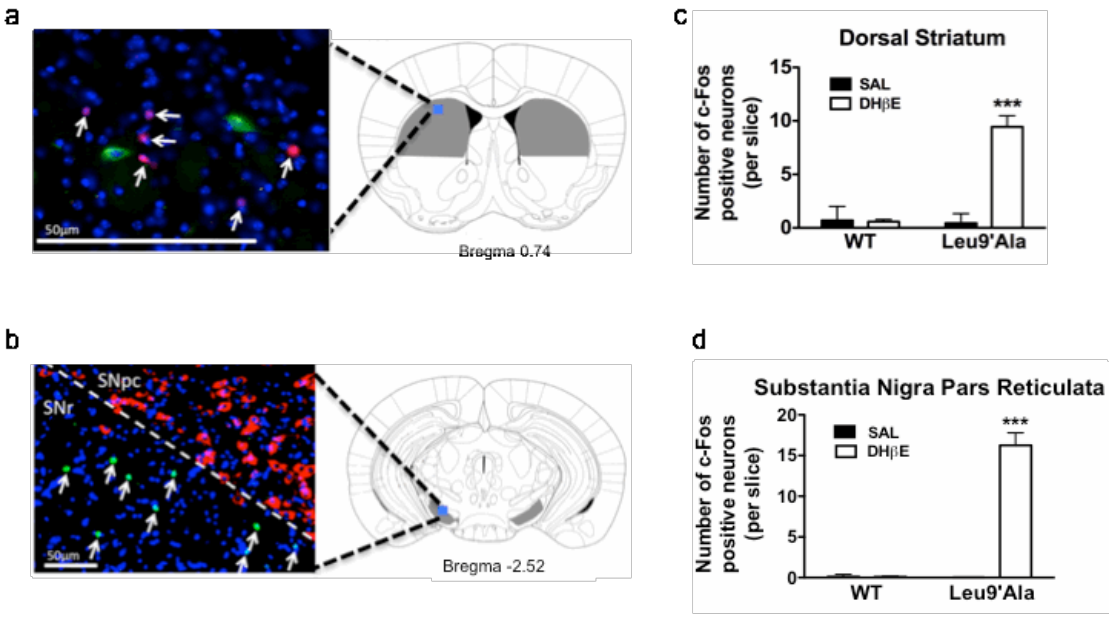

Figure II-5. Neuronal activation by DHβE in the dorsal ST and SNr. WT and homozygous Leu9′Ala were perfused 150 minutes after a challenge of saline (i.p.) or DHβE (1 mg/kg, i.p.) and coronal sections (20 µm thick) from the dorsal ST or SNr were isolated and immunolabeled to detect c-Fos and ChAT expression (ST) or TH expression (SNr). a) Top, representative brain atlas picture of illustrating ST region analyzed. An immuno-labeled coronal section from Leu9'Ala mice after injection with DHβE depicting c-Fos (red) and ChAT (green) expression is shown. DAPI stained nuclei are labeled blue. Bottom, number of c-Fos immuno-positive neurons/slice in WT and Leu9′Ala mice after saline or DHβE injection (3 mice/ treatment, 10 slices/mouse). b) Top, representative brain atlas picture of illustrating SNr region analyzed. An immuno-labeled coronal section from Leu9'Ala mice after injection with DHβE depicting c-Fos (green) and TH (red) expression is shown. DAPI stained nuclei are labeled blue. c and d) quantification of c-Fos immuno-positive neurons in WT and Leu9'Ala after saline or DHβE (3 mice/ treatment, 10 slices/mouse). ***p<0.001

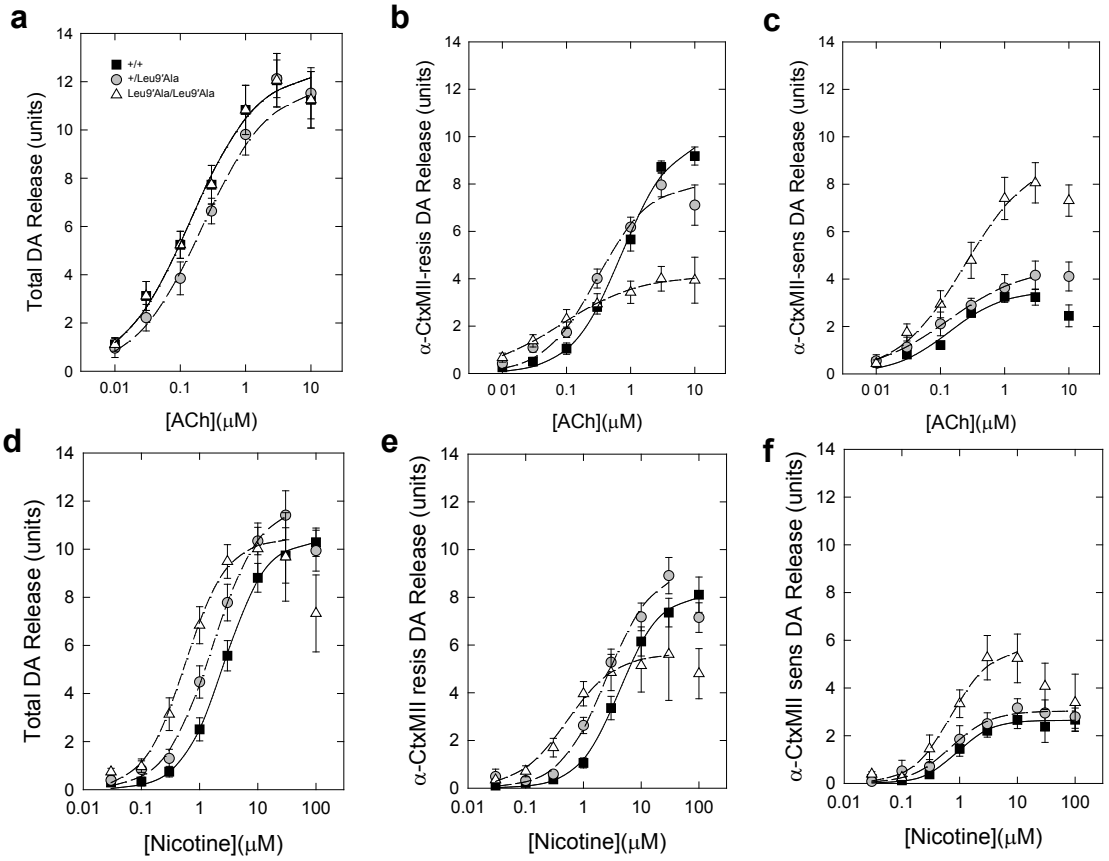

Figure II-6. Concentration response curves for ACh- and nicotine-stimulated [^{3}H]-DA release from striatal synaptosomes. a) Total ACh-stimulated [^{3}H]-DA release, b) $\alpha4\beta2$*-nAChR mediated [^{3}H]-DA release measured in the presence of α-CtxMII (50 nM) and c) $\alpha6\beta2$*-nAChR-mediated [^{3}H]-DA release determined by difference (a – b). Data represent means ± sem for n=9 +/+, n=8 +/Leu9′Ala and n=10 Leu9′Ala/Leu9′Ala mice. d-f) Analogous curves for nicotine-stimulated response. Data represent means ± SEM for n=8 mice of each genotype.

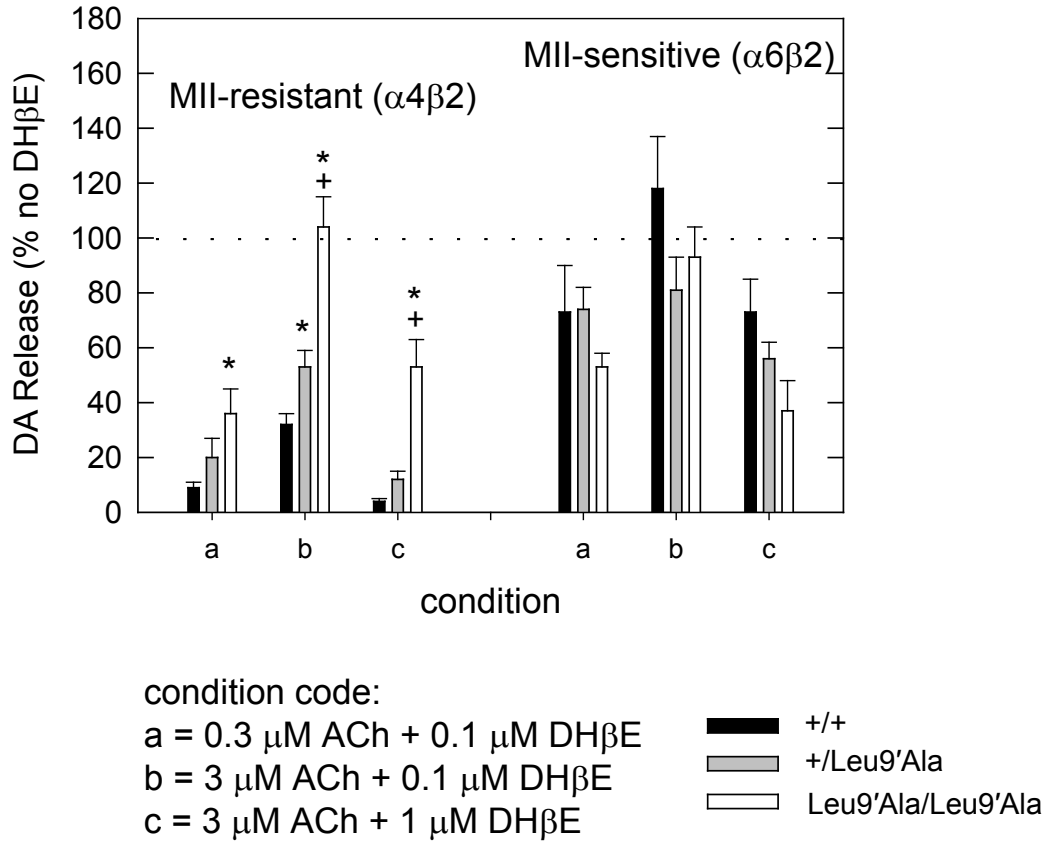

Figure II-7. Effect of DHβE on ACh-stimulated [^{3}H]-DA release. The α-CtxMII- resistant (α4β2*-nAChR-mediated) response in the homozygous Leu9′Ala is significantly less inhibited by DHβE (* different from WT) under the three conditions tested. The heterozygous Leu9′Ala were different only for condition b. No differences with genotype were seen for the effect of DHβE on the α-CtxMII-sensitive (α6β2*-nAChR mediated) portion of the response. All data expressed as % response in the absence of DHβE. Data represent means ± SEM for n=5 +/+, n=4 +/Leu9′Ala and n=3 Leu9′Ala/Leu9′Ala mice.

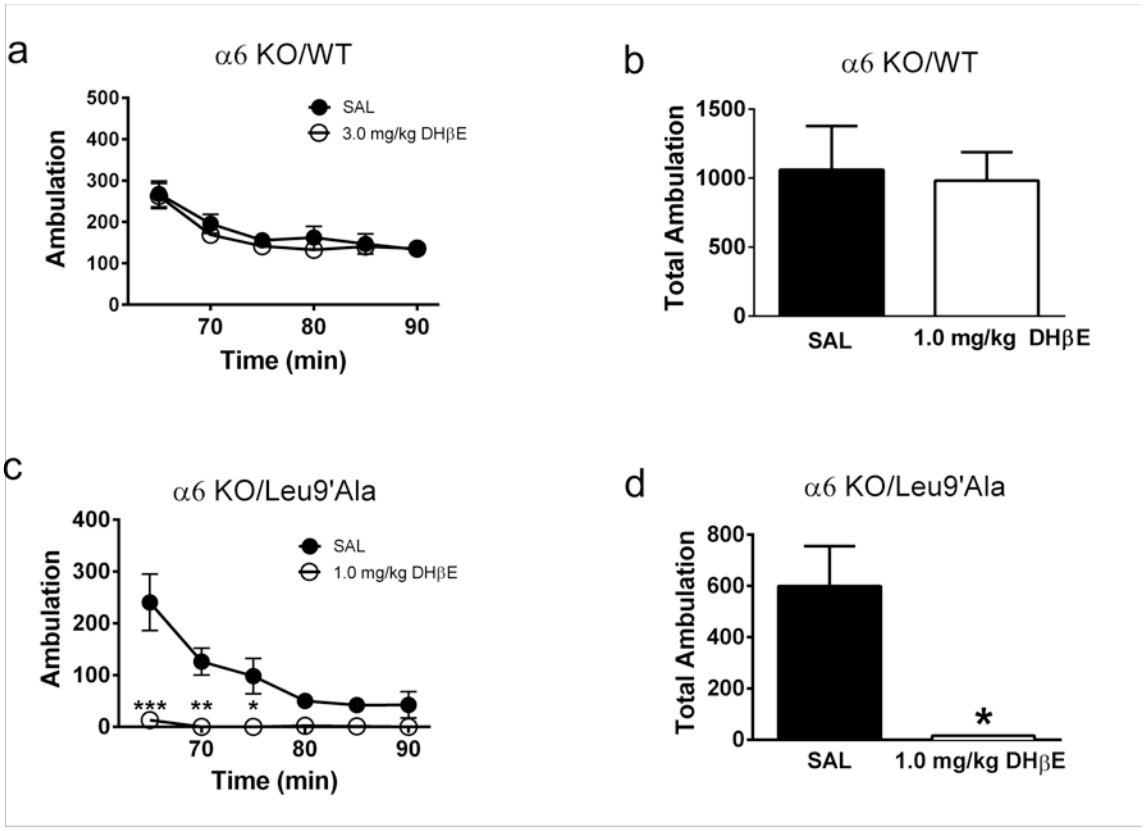

Figure II-8. α4(non-α6)β2* nAChRs mediate effect of DHβE in Leu9′Ala mice. a) α6KO/WT (n=8) mice were placed into novel cages 60 min after saline or DHβE challenge (1 mg/kg, i.p) and locomotor activity was measured for 30 minutes. Each data point represents the 5 min sum of ambulatory activity. b) Average summed ambulation over 30 min from panel a. c) α6KO/Leu9′Ala mice (n=2) were placed into novel cages 60 min after saline or DHβE challenge (1 mg/kg, i.p) and locomotor activity was measured for 30 minutes. Each data point represents the 5 min sum of ambulatory activity. b) Bar graphs represent averaged total ambulation during 30 min of locomotor activity in WT mice measured in panel a. *p<0.05, **p<0.01, and ***p<0.001.

Table II-1. Comparison of EC_{50} values for DA release

Genotype		EC_{50} (μM) for Total DA release	EC_{50} (μM) for $\alpha4\beta2$-mediated α-CtxMII-resistant	EC_{50} (μM) for $\alpha6\beta2$-mediated α-CtxMII-sensitive
+/+	ACh	0.41 ± 0.06	0.72 ± 0.11	0.14 ± 0.04
+/Leu9′Ala	ACh	0.22 ± 0.07 *	0.30 ± 0.06 *	0.13 ± 0.11
Leu9′Ala/Leu9′Ala	ACh	0.14 ± 0.05 *	0.08 ± 0.05 *+	0.24 ± 0.15
+/+	Nic	2.59 ± 0.13	4.16 ± 0.33	0.91 ± 0.12
+/Leu9′Ala	Nic	1.66 ± 0.15 *	1.74 ± 0.36 *	0.71 ± 0.16
Leu9′Ala/Leu9′Ala	Nic	0.57 ± 0.10 *+	0.52 ± 0.12 *+	0.73 ± 0.24

*Significantly different from +/+
+Significantly different from +/Leu9′Ala

Table II-2. Comparison of R_{max} values for ACh and nicotine

Genotype		R_{max} (units) Total DA release	R_{max} (units) $\alpha4\beta2$-mediated α-CtxMII-resistant	R_{max} (units) $\alpha6\beta2$-mediated α-CtxMII-sensitive
+/+	ACh	12.76 ± 0.56	10.76 ± 0.56	3.53 ± 0.30
+/Leu9′Ala	ACh	11.94 ± 1.02	8.06 ± 0.52 *	4.55 ± 0.97
Leu9′Ala/Leu9′Ala	ACh	12.51 ± 1.07	4.16 ± 0.06 *+	9.22 ± 1.68 *+
+/+	Nic	10.40 ± 0.13	8.16 ± 0.19	2.65 ± 0.08
+/Leu9′Ala	Nic	11.90 ± 0.33 *	9.19 ± 0.57	3.04 ± 0.13
Leu9′Ala/Leu9′Ala	Nic	10.50 ± 0.50 +	5.66 ± 0.41 *+	5.72 ± 0.68 *+

Units are (cpm released- baseline cpm)/(baseline cpm).
*Significantly different from +/+
+Significantly different from +/Leu9′Ala

Table II-3. Comparison of IC_{50} and K_i values (nM) for DHβE

Genotype	IC_{50} (nM) α4β2-mediated α-CtxMII-resistant using 3 μM ACh	IC_{50} (nM) α4β2-mediated α-CtxMII-resistant using 0.3 μM ACh	IC_{50} (nM) α6β2-mediated α-CtxMII-sensitive using 3 μM ACh	IC_{50} (nM) α6β2-mediated α-CtxMII-sensitive using 0.3 μM ACh
+/+	60 ± 4	11 ± 1	1848 ± 848	238 ± 169
+/Leu9′Ala	95 ± 23	12 ± 4	1175 ± 358	180 ± 49
Leu9′Ala/Leu9′Ala	1175 ± 412 *+	68 ± 12 *+	714 ± 140	118 ± 35
	K_i (nM) α4β2-mediated α-CtxMII-resistant using 3 μM ACh	K_i (nM) α4β2-mediated α-CtxMII-resistant using 0.3 μM ACh	K_i (nM) α6β2-mediated α-CtxMII-sensitive using 3 μM ACh	K_i (nM) α6β2-mediated α-CtxMII-sensitive using 0.3 μM ACh
+/+	12 ± 1	8 ± 1	83 ± 38	77 ± 55
+/Leu9′Ala	9 ± 2	6 ± 2	42 ± 13	49 ± 13
Leu9′Ala/Leu9′Ala	31 ± 11 *+	14 ± 3 *+	53 ± 10	39 ± 12

* significantly different from +/+. + significantly different from +/Leu9′Ala.

Notes: Data collected at 3 μM ACh from 5 WT (+/+), 4 heterozygous (+/Leu9′Ala), 2 homozygous (Leu9′Ala/Leu9′Ala) mice and at 0.3 uM ACh from 5 WT, 4 heterozygous, 3 homozygous mice. For each, 4-5 concentrations of DHβE were assayed, 0, 0.01, 0.1, 1, 10 μM with and without 50 nM αCtxMII. Data was curve fit from all individual points for each ACh concentration using the single exponential decay equation: f=a*exp(-b*x), where a=uninhibited release, b=decay constant and 0.693/b=IC_{50} value. The K_i values were calculated from the equation: $K_i=IC_{50}/(1+[ACh]/EC_{50})$ where EC_{50} values are taken from Table II-1. By ANOVA, using all determinations of K_i, the resistant K_i for heterozygous and homozygous is significantly different than WT.

CHAPTER III.

DHβE INDUCES A MOTOR PHENOTYPE AND AN AVERSIVE STATE IN MICE CONTAINING HYPERSENSITVE α4* NICOTINIC ACETYLCHOLINE RECEPTORS

Contributions to Chapter III

This chapter is not published.

Author Contributions

Soll LG – genotyping, designed and performed experiments, performed data analysis, prepared figures, and wrote this chapter.

Guildford MJ, Ngolab J, and Pang X – genotyping

Tapper AR and Gardner PD – designed experiments data analysis and edited chapter.

ABSTRACT

Dopamine (DA) is the primary neurotransmitter involved in the motor and reward pathway. Depletion of DA concentrations in the dorsal striatum can lead to movement disorders while depletions in the ventral striatum, also known as the nucleus accumbens (NAc), are associated with withdrawal. Nicotinic acetylcholine receptors (nAChRs) can modulate DA release, thus may play a large role in motor and withdrawal-associated behaviors. We previously reported that mice containing hypersensitive α4* nAChRs (Leu9′Ala) challenged with DHβE, an α4* nAChR antagonist, exhibit robust motor deficits characterized by hypolocomotion, akinesia, catalepsy, tremors, and hindpaw clasping. Here we investigate the effects of DHβE in Leu9′Ala heterozygous mice which induces a less robust phenotype (characterized by hypolocomotion, akinesia, myoclonic jerk, and hindpaw clasping). In addition to motor symptoms, these mice also condition a place aversion to DHβE and exhibit decreased locomotor activity in response to DHβE (before other motor deficits are apparent). These behaviors are normally associated with nicotine withdrawal. We hypothesize that DHβE decreases DA levels in the NAc, through α4* nAChRs, to induce a withdrawal like syndrome, in the absence of chronic nicotine exposure. This suggests that α4* nAChRs may be potential therapeutic targets to treat movement deficits and nicotine withdrawal.

III.A. Introduction

Dopamine (DA) levels in the striatum regulate motor output in the basal ganglia motor circuit (Rice et al., 2011). Nicotinic acetylcholine receptors (nAChRs), located on cell bodies and terminals of DAergic neurons that project from the substantia nigra pars compacta (SNpc) to the striatum, mediate DA release by responding to acetylcholine (ACh) provided by cholinergic signaling to the SNpc and cholinergic interneurons in the striatum (Grady et al., 2007, Albuquerque et al., 2009). In striatal slices, electrically-evoked DA release decreased by 90% after acetylcholine (ACh) depletion or nAChR antagonism (Zhou et al., 2001). Experiments in striatal synaptosomes reveal that the majority of nicotine-mediated DA release in the striatum is mediated through presynaptic α4* nAChRs and the minority (about 30%) is mediated by α6* nAChRs (Salminen et al., 2004, Threlfell et al., 2012). These experiments suggest that nAChRs play a major role in regulating striatal DA release; therefore, these receptors likely modulate motor output. However, pharmacological blockade of nAChRs or genetically deleting these receptors has little significant effect on motor output (Ross et al., 2000, Champtiaux et al., 2002). As a result, elucidating the functions of nAChRs on motor output has been difficult. Fortunately, mice expressing hypersensitive α4* or α6* nAChRs may be used to elucidate the roles of these receptors in motor activity. Transgenic mice expressing gain-of-function α6* nAChRs are spontaneously hyperactive (Drenan et al., 2010). Knock-down

of the α4 subunit in these mice reduced locomotor activity back to levels measured in WT mice, suggesting that the α4α6β2* nAChR is important for movement (Drenan et al., 2010). I have previously reported that α4(non-α6)* nAChRs mediate a robust, transient motor deficit, characterized by hypolocomotion, akinesia, catalepsy, and rigidity, and tremors, induced by antagonizing α4* nAChRs with DHβE, a selective α4β2* nAChR antagonist, in knock-in mice with enhanced sensitivity to ACh due to a mutation in the α4 subunit (Leu9′Ala) (Chapter II). I hypothesized that antagonism of these receptors caused a major depression of DA levels, which generated significant motor deficits. Challenging Leu9′Ala homozygous mice with amphetamine or a DA D_1 receptor agonist prior to DHβE treatment prevented the DHβE-induced motor phenotype, supporting the hypothesis that the phenotype is DA-dependent.

NAChRs regulate other DA signaling pathways, most notable is their role in the reward pathway involved in nicotine reward, addiction, and withdrawal (De Biasi and Salas, 2008, Albuquerque et al., 2009). Using Leu9′Ala homozygous mice, α4* nAChRs were shown to be sufficient for nicotine reward, which is associated with increased DA concentrations in the nucleus accumbens (NAc) (Tapper et al., 2004, Markou, 2008). Local infusion of DHβE into the VTA blocks nicotine-self administration, which is important for nicotine dependence (Watkins et al., 1999). Nicotine withdrawal, associated with decreased accumbal DA

concentrations, can be elicited with DHβE in nicotine dependent mice (Hildebrand et al., 1999, Watkins et al., 2000). The aversive nicotine withdrawal state is measured by conditioned place aversion, fear conditioning, anxiety, and reduced reward (Epping-Jordan et al., 1998, Watkins et al., 2000, Bruijnzeel and Markou, 2004, Kenny and Markou, 2005, Jackson et al., 2008, Portugal et al., 2008, Stoker et al., 2008). Together, these results suggest that α4* nAChRs modulate nicotine reward and withdrawal behaviors through DA signaling.

Given that α4* nAChRs modulate DA release throughout the brain, I hypothesize that antagonism of hypersensitive α4* nAChRs in Leu9′Ala mice will result in a decreased DA release. To test this hypothesis, I tested motor and withdrawal-related behaviors in DHβE-challenged Leu9′Ala and WT mice. Here, I show that blocking α4* nAChRs in Leu9′Ala heterozygous mice produces a motor phenotype characterized by hypolocomotion, myoclonic (involuntary) jerks, akinesia, and hindlimb clasping, and also a withdrawal-like state measured by decreased locomotor activity and conditioned place aversion. Both hypolocomotion and aversion are often associated with lowered DA levels, suggesting that blockade of Leu9′Ala α4* nAChRs can be used as a model to study α4* nAChRs in DA signaling-related behaviors.

III.B. Materials and Methods

Animals In all experiments, female Leu9′Ala knock-in and wild-type littermate (WT) mice were used. These mice have been backcrossed to C57BL/6 mice for at least 9 generations. The genetic engineering of the Leu9′Ala line has been previously described (Tapper et al., 2004). Within the animal facilities at the University of Massachusetts Medical School under a 12h light: 12 h dark lighting cycle, mice bred, housed with no more than 5 mice per cage, and receive food and water *ad libitum*. All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council (National Research Council, 1996) as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Drugs Dihydro-β-erythroidine hydrobromide (DHβE) was purchased from Tocris Bioscience Bristol, UK. DHβE was dissolved in a 0.9% saline and administered via an intraperitoneal (i.p.) injection at a dose of 3 mg/kg.

Locomotor Activity For all experiments measuring locomotor activity, mice were given saline injections once a day for 3 days prior to the experiment to reduce differences in locomotor activity due to stress from the injection and handling. On the day of the experiment, mice were habituated to the room for about 40

minutes. Locomotor chambers equipped with infrared photobeams (San Diego Instruments) were used to measure locomotor activity by quantifying the number of beam breaks. Mice were given saline (i.p.) or DHβE (3 mg/kg, i.p.) and placed into novel cages within locomotor chambers at the time points indicated and measured for 30 minutes with beam breaks summed every 5 minutes. Mice served as their own control such that the first experiment half of the mice received saline and the other half received DHβE in the first round of locomotor activity. Then locomotor activity was measured again the following week in the same group of mice. Mice that received saline the first week were administered DHβE and mice that already received DHβE were administered saline.

Motor Characterizations Motor characterizations were previously described in Soll et al. 2013. First, mice were habituated to a novel cage before a battery of motor characterizations was conducted. Over a time course of 180 minutes, myclonic jerk, akinesia, catalepsy, clasping, and tremor were measured at the indicated time points. Prior to administration of saline (i.p.) or DHβE (3 mg/kg, i.p.), the motor characterizations were measured (“0” time point).

Myoclonic Jerk Before mice were handled for the characterization tests (described below), mice were observed for 30 seconds in the habituated cage. During the 30-second time period, the number of myclonic jerks was counted. This test was conducted every 30 minutes.

Akinesia In an empty cage, mice were lifted by the tail so that forelimbs remained on the floor. Two trials were conducted, each lasting 30 seconds, counting the number of forepaw steps. This test was conducted every 30 minutes and the two trials were averaged for each time point.

Catalepsy Following the akinesia test, a raised bar 5 centimeters from the floor was placed within the same empty cage. The forelimbs of the mouse were placed on the bar and latency to remove them was measured for up to 2 minutes. This was measured every 2 hours.

Clasping and Tremor Mice were raised by their tail and tremor and clasping were evaluated over 10 seconds. A score was given to depict the degree of clasping or body tremor occurred. The scoring for clasping was as follows: 0 = hind limbs spread wide apart (normal position), 1 = hind limbs 25% closed, 2 = hind limbs 50% closed, 3 = hind limbs 75% closed with periods of hind limbs clasped, 4 = hind limbs fully clasped for 10 seconds. The severity of a body tremor was scored: 0 = no tremor, 1 = isolated twitches, 2 = intermittent tremors, 3 = continuous tremor.

Conditioned Place Aversion Conditioned place aversion (CPA) is used to test for a drug's ability to induce a negative state. Located within a sound attenuation

chamber to avoid outside disturbances, the CPA apparatus (model ENV-3013, Med Associates, Inc.) contains 2 chambers separated by a neutral compartment with guillotine doors for access to either chamber. All 3 compartments contain infrared photobeams to record activity, which is recorded by MED-PC software. The smaller, central, neutral compartment is grey with smooth PVC flooring and guillotine doors to access the neighboring chambers. These neighboring chambers are larger and differ from each other in color (white or black) and metal flooring (mesh or rod). The neutral compartment is grey with smooth PVC flooring. Before CPA experiments, all mice were exposed to an i.p. injection of saline and placed in the room with the CPA chamber for about 30 minutes with the sound attenuation fan on for habituation purposes. During pre-conditioning, conditioning, and test days, mice were also habituated to the room with the sound attenuation fan on for at least 30 minutes. On the pre-conditioning day, drug-naïve mice were placed into and confined to the central grey chamber (neutral) for 5 minutes. After this time, the guillotine doors were manually lifted and mice were allowed to roam freely between a white and dark chamber for a 30-minute period to assess baseline preference and time spent in each chamber. For the following 2 conditioning days, mice were trained to associate each chamber with a drug. In the morning of each conditioning day, each mouse was administered saline (i.p.) and immediately confined to the non-preferred chamber (determined on pretest day) for 30 minutes. In the afternoon, mice were administered DHβE (i.p., 3 mg/kg) and immediately confined to the preferred

chamber for 30 minutes. On test day, mice were initially placed into and restricted to the grey chamber for 5 minutes. After the 5-minute restriction period, the doors were lifted and mice were free to roam all chambers for 30 minutes and time spent in each chamber was measured. A difference score was calculated as the time spent in the saline- or DHβE-paired chamber on test day minus habituation day. Decreased time spent in the DHβE-paired chamber on the test day compared to pre-test day indicated aversion.

Data Analysis Behavioral data were analyzed with t-test, one or two-way ANOVA with repeated measures, as indicated. Post-hoc analysis was done using Bonferroni post-hoc tests. Data were analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

III.C. Results

Our previous study revealed that hypolocomotion along with akinesia, catalepsy, and tremor in Leu9′Ala mice occurred after blocking α4* nAChRs (Soll et al., 2013). To test if a similar motor phenotype could be induced by DHβE in Leu9′Ala heterozygous mice, we compared locomotor activity and motor behaviors including myoclonic jerk, akinesia, catalepsy, clasping, and tremor (Fig. III-1) in Leu9′Ala heterozygous and wild-type littermate (WT) mice challenged with saline or DHβE. Indeed, DHβE induced an intermediate motor

phenotype in Leu9′Ala heterozygous mice compared to their homozygous counterparts. Upon a 5 minute observation staring at 90 minutes after DHβE challenge, WT mice revealed no effect of drug, but Leu9′Ala mice had temporary bouts of being “frozen” in place and exhibited an abnormal posture, straub tail (rigid and erect), rigidity, and clasped hind paws (data not shown). To characterize deficits in motor behaviors induced by DHβE in Leu9′Ala heterozygous mice, measurements for myoclonic jerk, akinesia, catalepsy, clasping, and tremor were taken at specific times over 3-hours. Analysis by a two-way repeated measures ANOVA of myoclonic jerks (Fig. III-1a) indicated a significant effect of time ($F_{6,84}$ = 4.888, p=0.0002), genotype ($F_{1,84}$ = 20.46, p= <0.0001) and an interaction between time and genotype ($F_{6,84}$ = 4.888, p=0.0002). Bonferroni’s post-hoc analysis indicated DHβE significantly increased the number of myoclonic jerks in Leu9′Ala heterozygous compared to WT mice at 90 (p=0.0002) and 120 minutes (p<0.0001). Two-way repeated measures ANOVA analysis of akinesia (Fig. III-1b) revealed a significant effect of time ($F_{6, 133}$ = 6.727, p<0.0007) and genotype ($F_{6, 133}$ = 10.29, p=0.0017). Although there was not a significant cataleptic effect of DHβE (Fig. III-1c), there was a trend toward increased catalepsy in Leu9′Ala heterozygous mice compared with WT at 120 minutes analyzed by Bonferroni’s post hoc analysis. Analysis of clasping (Fig. III-1d) by two-way repeated measures ANOVA, indicated a significant effect of genotype ($F_{6,119}$ = 44.61, p<0.0001), time ($F_{6, 119}$ = 8.257, p<0.0001), and interaction between genotype and time ($F_{6, 119}$= 8.257,

p<0.0001). Further post-hoc analysis by Bonferroni's test revealed that DHβE induced significant increases in clasping of Leu9′Ala heterozygous compared to WT mice at 90 (p<0.0001) and 120 minutes (p<0.0001). DHβE did not induce tremors in WT or Leu9′Ala heterozygous mice (Fig. III-1g).

Locomotor activity was measured 60 minutes after administration of saline (i.p.) or DHβE (3 mg/kg i.p.) in WT and Leu9′Ala heterozygous mice to test for differences in ambulation after α4* nAChR blockade (Fig. III-2). WT mice exhibited no changes in activity level in either the locomotor activity time course (Fig. III-2a) or total ambulation over 30 minutes (Fig. III-2b). There was a significant reduction in locomotor activity in Leu9′Ala heterozygous mice in both the time course and total ambulation. Two-way repeated measures ANOVA analysis of locomotor time course (Fig. III-2c) revealed a significant effect of drug treatment ($F_{1,90}$ = 52.31, p<0.0001) and time ($F_{5,90}$ = 8.475, p<0.0001). Bonferroni post-hoc analysis indicated that DHβE induces hypolocomotion 60 minutes post-injection with statistical significance at 65 (p<0.05), 70 (p<0.001), 75 (p<0.001), 80 (p<0.05), and 85 (p<0.05) minutes. A paired t-test analyzing total ambulation over 30 minutes in Leu9′Ala mice (Fig. III-2d) revealed that DHβE induced significant hypolocomotion 90 minutes after administration (p<0.05). No significant differences in basal activity levels were measured between Leu9′Ala heterozygous and WT mice (data not shown).

CPA and hypolocomotion are common features of a drug withdrawal state in rodents and are associated with low DA levels in the NAc, a brain region within the reward circuitry (Laviolette and van der Kooy, 2004, De Biasi and Salas, 2008). To test the hypothesis that α4* nAChR blockade in Leu9′Ala heterozygous mice induces a negative withdrawal like syndrome, we tested for a CPA and locomotor activity (prior to emergence of motor symptoms which occur 90 minutes post challenge) (Fig. III-3). Drug-free mice were able to freely roam in a CPA apparatus before and after conditioning of saline (i.p.) or DHβE (3 mg/kg, i.p.) to specific chambers. Decreased time spent in the chamber after conditioning compared to pre-conditioning is associated with aversion. WT mice (Fig. III-3a) did not condition a place aversion to DHβE, however Leu9′Ala heterozygous (Fig. III-3b) displayed a significant aversion to the drug as indicated by a paired t-test ($p<0.05$).

Locomotor activity was measured 15 minutes after administration of saline (i.p.) or DHβE (3 mg/kg, i.p.). Motor deficits, such as akinesia and catalepsy, were not apparent at this time point. Locomotor activity was unchanged by DHβE compared with a saline injection in WT mice (Fig. III-3c and e). However Leu9′Ala heterozygous mice displayed depressed locomotor activity (Fig. III-3d and f). Two-way repeated measures ANOVA analysis of the locomotor activity time course (Fig. III-3e) revealed a significant effect of drug treatment ($F_{1, 65}$ = 36,86, $p<0.0001$) and genotype ($F_{4,65}$ = 16.93, $p<0.001$). There was a significant

decrease of locomotor activity at 25 ($p<0.05$), 30 ($p<0.05$), and 35 ($p<0.05$) minutes. Total ambulation (Fig. III-3f) was also significantly decreased ($p<0.05$).

III. D. Discussion

DHβE induces a motor deficit in Leu9'Ala heterozygouserozygous mice

We previously described that antagonism of hypersensitive α4* nAChRs in knock in (Leu9′Ala) mice induced a reversible, robust motor phenotype characterized by hypolocomotion, akinesia, catalepsy, tremor, and hind limb clasping (Soll et al., 2013). However, these mice had a very robust, saturating phenotype and may consequently limit its potential as a model to investigate α4* nAChRs role in spontaneous motor behavior**.** I hypothesize that blocking α4* nAChRs in the Leu9′Ala heterozygous mice will result in an intermediate phenotype associated with decreases in DA levels. Since mice are only heterozygous for hypersensitive receptors, reductions in DA levels should not be as steep as hypothesized to occur in Leu9′Ala homozygous mice, thus resulting in a phenotype in heterozygous mice that is less robust. This potentially would allow us to study finer modifications to motor behaviors after further pharmacological perturbation, rendering these mice a better alternative model than using Leu9′Ala homozygous mice to study the role of α4* nAChRs in motor output.

Indeed, an intermediate phenotype was induced by DHβE in Leu9′Ala heterozygous mice but no deficits were revealed in WT mice after challenge with DHβE. Abnormal freezing movements, straub tail, and abnormal posture were observed in DHβE challenged Leu9′Ala heterozygous mice. Additionally I measured increased akinesia and myoclonic jerks as well as a robust clasping phenotype. In a time-course, I found that these motor characterizations were strongest at approximately 90 minutes and were alleviated about 3 hours later, demonstrating that the phenotype is reversible. Leu9′Ala heterozygous mice have a stronger clasping phenotype compared with homozygous mice, probably as a result of decreased catalepsy which could limit range of motion needed for clasping in homozygous mice (Soll et al., 2013). Myoclonic jerks were only present in heterozygous mice. In addition, a tremor response to DHβE is not apparent in Leu9′Ala heterozygous mice, but was previously recorded in Leu9′Ala homozygous mice (Soll et al., 2013).

The DHβE-induced motor deficits were debilitating in Leu9′Ala homozygous mice, which was most apparent in DHβE's abolition of locomotion measured at 60 minutes (Soll et al., 2013). However, in Leu9′Ala heterozygous mice locomotor activity is significantly decreased but still some levels of locomotor activity are retained. Once again, this resembles a less robust phenotype than exhibited by DHβE-challenged Leu9′Ala homozygous mice. It is interesting to note that both Leu9′Ala homozygous and heterozygous mice do not have

differences in basal levels of activity when compared to WT mice. This suggests that there is some type of compensation occurring, most likely a downregulation of Leu9′Ala α4* nAChRs, so that the hyperactivity of the α4* nAChRs do not affect overall locomotor activity in a drug naïve Leu9′Ala mice.

One interesting aspect of this phenotype is the time frame in which it occurs. Measured by decreased locomotor activity, the onset of the phenotype emerges around 60 minutes with the most robust effects transpiring at 90 minutes and lasting until 120 minutes until they begin to dissipate around 3 to 4 hours. Our model mimics the onset and reversal of akinesia after blockade of the medial forebrain bundle by tetrodotoxin in mice (Galati et al., 2009). Microdialysis measured decreases in striatal DA levels as low as approximately 25% of baseline. Therefore, akinesia in tetrodotoxin-challenged mice results from low striatal DA levels. Since motor symptoms are occurring within the same time frame, it supports our hypothesis that DA levels are decreased in Leu9′Ala mice challenged with DHβE.

A withdrawal like state is induced in drug-naïve Leu9′Ala heterozygous mice by blocking α4 nAChRs*

A primary component of the reward circuitry is the DAergic projection from the ventral tegmental area (VTA) to the NAc. Rewarding drugs increase DA levels in the NAc, while decreases in DA are measured after spontaneous or precipitated withdrawal in rodents (Fung et al., 1996, Hildebrand et al., 1998, Rada et al., 2001, Gaddnas et al., 2002, Rahman et al., 2004). Furthermore, α4 KO mice do not self-administer nicotine, but re-expression of α4* nAChRs in the VTA, but not the SNpc, rescues this behavior, implying a role for VTA α4* nAChR in nicotine-evoked DA release in the NAc (Pons et al., 2008). Low DA levels in the NAc are thought to bring about withdrawal behaviors in nicotine dependent rodents (Hildebrand et al., 1999, Watkins et al., 2000). DHβE can induce an affective withdrawal syndrome, classified by anxiety, CPA, and hypolocomotion, in nicotine-dependent mice (Damaj et al., 2003, Jackson et al., 2009a). To test our hypothesis that DHβE induces a widespread lowered DA state in Leu9′Ala mice, we tested behaviors associated with affective withdrawal. If observed, these results would imply that changes in DA levels occur in DA signaling pathways outside the motor circuit. Despite the fact the drug-naïve WT mice were unable to condition a place aversion to DHβE, drug-naïve Leu9′Ala heterozygous mice exhibited a CPA response. Additionally, locomotor activity was decreased by DHβE in Leu9′Ala heterozygous mice but not WT. This was tested at a time

period prior to onset of motor symptoms such as clasping, rigidity, straub tail, and myoclonic jerks, suggesting that hypolocomotion is associated with an affective state and not a motor deficit. Direct infusion of mecamylamine into the VTA of nicotine-dependent rats will condition a place aversion and decrease locomotor activity (Hildebrand et al., 1999). This confirms that both CPA and hypolocomotion measured at 30 minutes are reflective of an aversive state modulated by the primary circuitry involved in reward and withdrawal and not a direct effect of the primary motor circuit.

Further investigation is needed to truly assess changes in reward as a result of a withdrawal state in Leu9′Ala mice challenged with DHβE. The most direct measurement of reward is intracranial self-stimulation (ICSS). Reward deficits were measured through elevated ICSS levels after either spontaneous withdrawal or mecamylamine-precipitated withdrawal from nicotine in mice (Johnson et al., 2008). It is suggested that the ICSS test not only assesses decreased reward, but may reflect the negative reinforcement of nicotine that drives drug seeking behaviors and impedes smoking cessation (Koob et al., 2004, Johnson et al., 2008).

One advantage of studying the effects of DHβE in Leu9′Ala mice is that chronic nicotine treatment necessary to induce dependence in WT mice is time consuming, lasting from a few weeks to even as long as 2 or more months

depending on the method of nicotine administration (Pietila et al., 1998, Isola et al., 1999, Damaj et al., 2003). Our model utilizes an inherent heightened dependency on basal ACh levels to evaluate behaviors after blocking these receptors in drug-naïve mice, thereby bypassing the need to chronically treat mice to induce changes required for dependency and withdrawal. Although this is not an ideal model for nicotine withdrawal, it may be used to identify the affective behaviors modulated by α4* nAChRs and to further elucidate the mechanism of this modulation.

A model for α4 nAChR mediation of a lowered DA state*

I propose that there is a significance to the progression of DA-related behaviors over time in our model. I hypothesize that the motor and aversive behaviors in Leu9′Ala mice after DHβE challenge are associated with reductions in the DA levels in the striatum and NAc occurring at different times. As in our model, an affective state measured by CPA and hypolocomotor activity occurs before the onset of the motor symptoms. This progression of symptoms is mimicked in Parkinsons's Disease (PD) where an affective state consisting of anxiety and depression is thought to precede motor symptoms (PD) (Shiba et al., 2000, Ishihara and Brayne, 2006, Jacob et al., 2010, Poletti et al., 2012). NAChR upregulation in the substantia nigra pars compacta has been measured in mild to moderately lesioned mice that do not exhibit hypolocomotor behavior, suggesting

that nAChR upregulation leads to increased DA release in order to delay onset of motor symptoms (Kryukova et al., 2013). A similar upregulation, especially of α4* nAChRs, occurs in response to chronic nicotine exposure and may be a mechanism by which smoking has a neuroprotective effect against PD (Flores et al., 1992, Thacker et al., 2007, Quik et al., 2009, Chen et al., 2010). Furthermore α4β2* nAChRs are protected during nigrostriatal damage, suggesting these receptors may be good therapeutic targets for increasing DA levels (Bordia et al., 2007, Quik et al., 2009).

The Leu9′Ala mouse model could be a unique system to study α4* nAChRs in several DA- related behaviors. In order to establish this model, we need to test our hypothesis that Leu9′Ala mice challenged with DHβE have decreased DA levels. The most direct and definitive method would be to use in vivo microdialysis to measure DA levels in the dorsal striatum and NAc over a time course and correlate the changes in DA levels with the onset and extinction of behaviors. Once we have established DA level change in Leu9′Ala mice challenged with DHβE, it will confirm that this model can be used to test for α4* nAChR modulation of DA-related behaviors. Further experiments, such as local infusion of DHβE or shRNA knockdown of α4* nAChRs, can be pursued to tease apart the mechanism of this modulation by distinct brain regions and even specific cell subtypes.

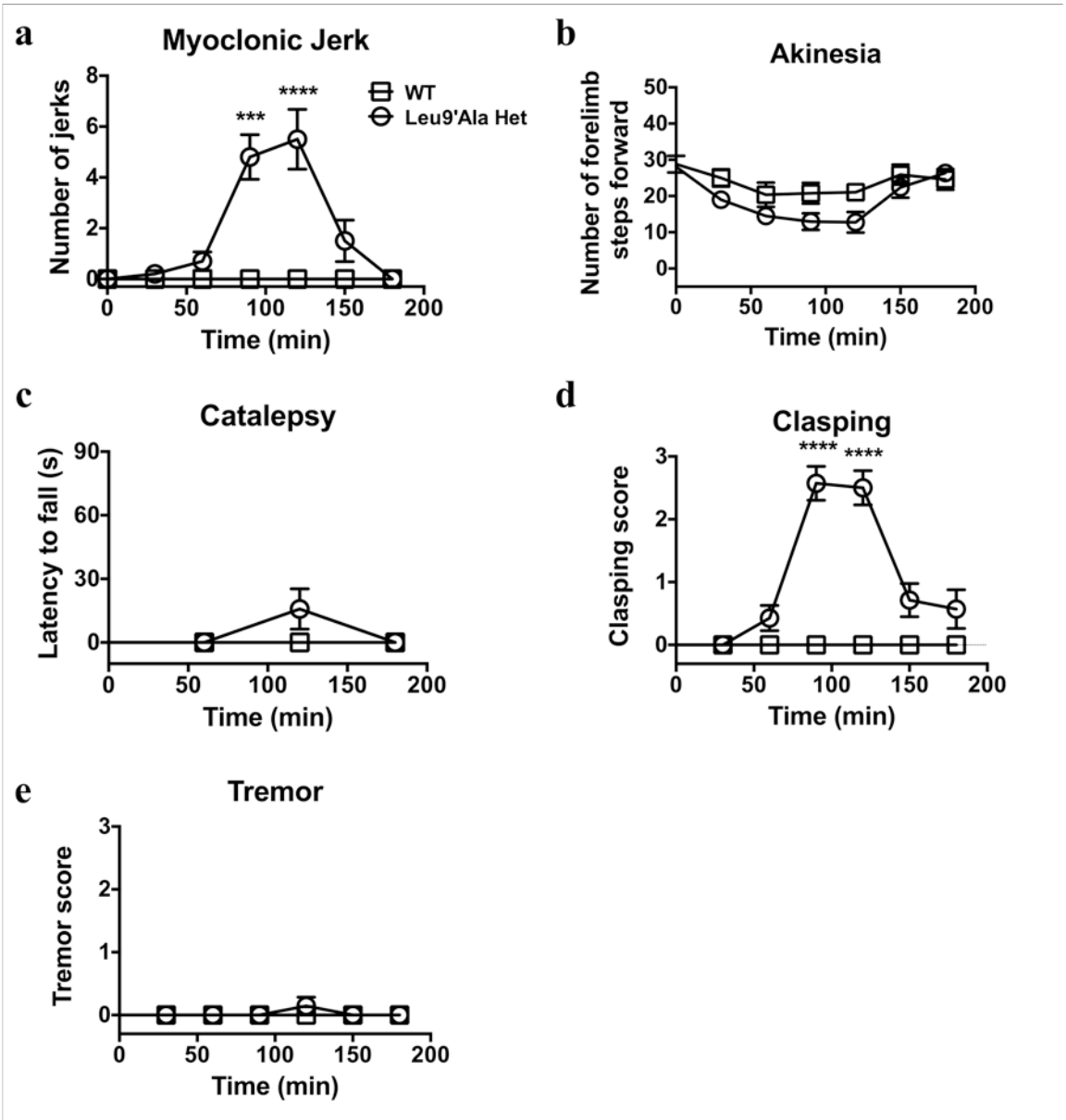
a
Myoclonic Jerk
Number of jerks
Time (min)
WT
Leu9'Ala Het
***
****
b
Akinesia
Number of forelimb steps forward
Time (min)
c
Catalepsy
Latency to fall (s)
Time (min)
d
Clasping
Clasping score
**** ****
Time (min)
e
Tremor
Tremor score
Time (min)

Figure III-1

Figure III-1. DHβE induces motor abnormalities in Leu9′Ala heterozygous mice. a-d) Characterization of motor symptoms in WT and Leu9′Ala heterozygous mice measured every 30 minutes over a 180-minute time period immediately following DHβE (3 mg/kg, i.p.). a) Myoclonic jerk: Number of myoclonic jerks were counted in 30 seconds (WT: n=5, Leu9′Ala heterozygous: 14). b) Akinesia: Number of forelimb steps forward were counted for 30 seconds (WT: n=4, Leu9′Ala heterozygous: n=14). c) Catalepsy: Latency for forelimbs to fall off a raised bar for 2 minutes was recorded (WT: n=5, Leu9′Ala: heterozygous n=14). d) Clasping of hind limbs during a 10-second period was measured (WT: n=3, Leu9′Ala heterozygous: n=14). e) tremor (WT: n=5, Leu9′Ala: heterozygous n=14)***$p < 0.001$, ****$p<0.0001$ analyzed by Bonferroni post-hoc test.

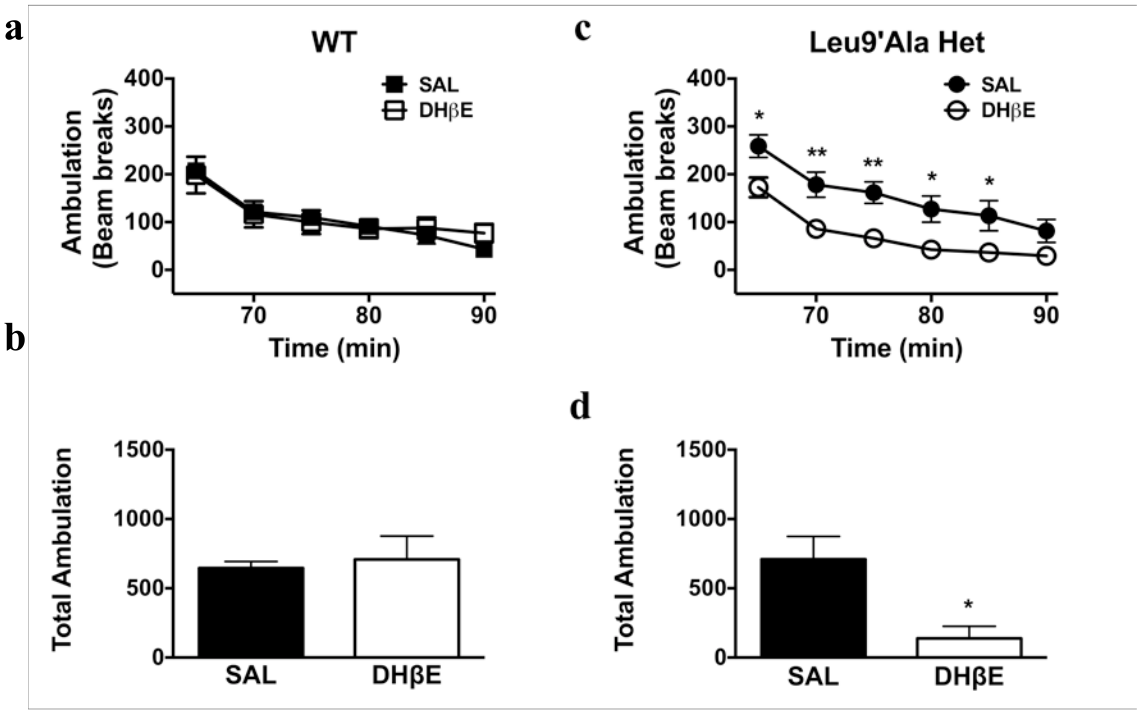

Figure III-2. DHβE decreases locomotor activity of Leu9′Ala heterozygous mice. a) WT mice were placed into novel cages 60 minutes after saline (i.p., n=4) or DHβE challenge (3 mg/kg, i.p, n=4) and locomotor activity was measured for 30 minutes. Each data point represents the 5-minute sum of ambulatory activity. b) Bar graphs represent total ambulation during 30 min of locomotor activity in WT mice measured in panel a. c) Locomotor activity in Leu9′Ala heterozygous mice starting 60 min after saline (i.p., n=7) or DHβE (3 mg/kg, i.p., n=7). d) Total ambulation was quantified for each condition as in panel b *$p<0.05$ and **$p < 0.01$ analyzed by Bonferroni's post-hoc test.

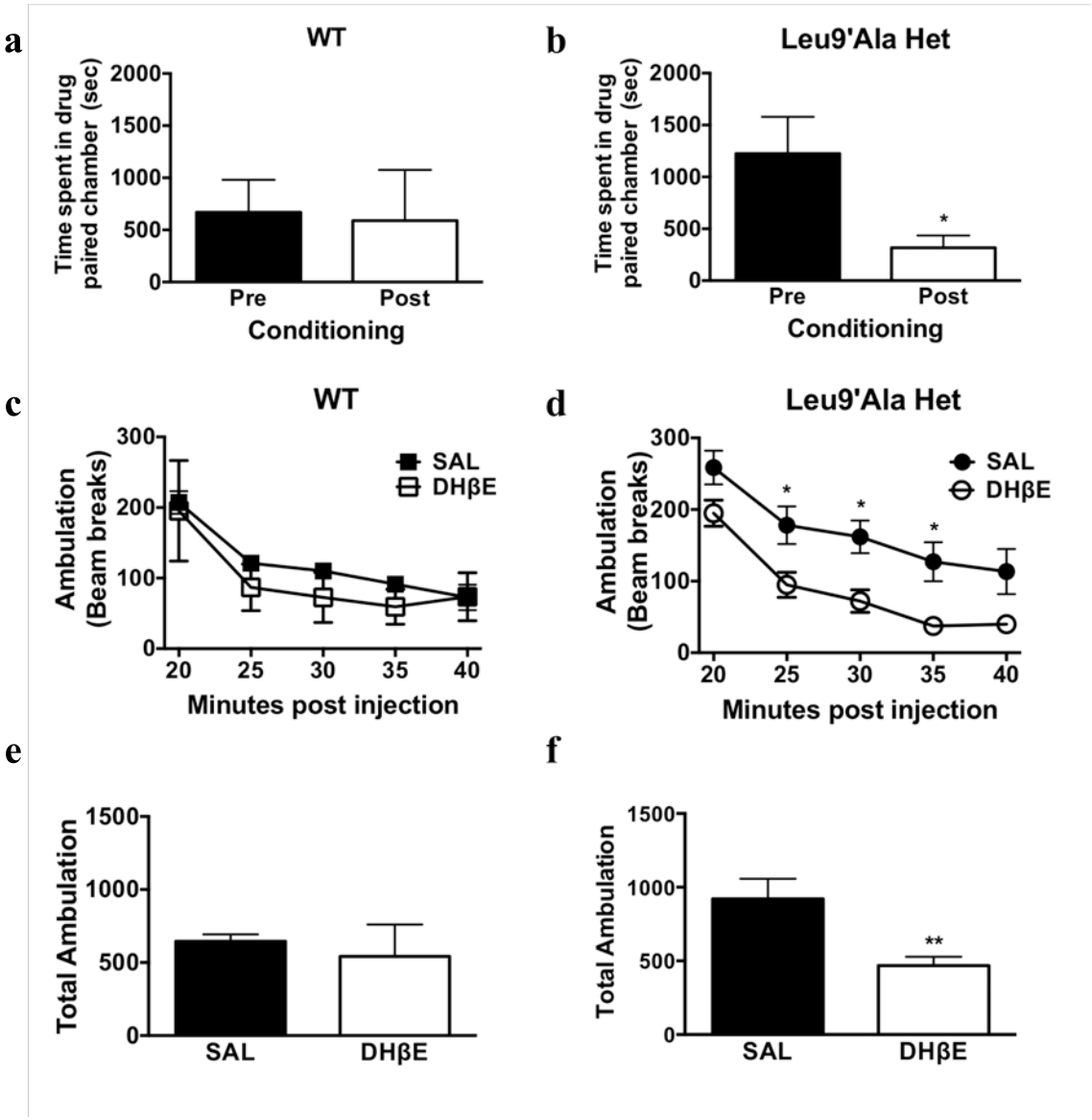

Figure III-3. A negative state is induced in DHβE-challenged Leu9′Ala heterozygous mice Time spent in the DHβE (3 mg/kg, i.p.) paired chamber before and after conditioning in a) WT (n=3) and b) Leu9′Ala heterozygous (n=4) mice in the conditioned place aversion assay. A time course of 30 minute locomotor activity was measured starting 15 minutes after challenge with saline (i.p.) or DHβE (3 mg/kg, i.p.) in c) WT (n=4) and d) Leu9′Ala heterozygous mice (n=7). Each data point represents the 5-minute sum of ambulatory activity. e and f) Total ambulation was quantified for WT and Leu9′Ala heterozygous mice, respectively. *p<0.05 and **p < 0.01 analyzed by Bonferroni's post-hoc test.

CHAPTER IV.

NICOTINIC ACETYLCHOLINE RECEPTORS CONTAINING AN α4 OR α6 SUBUNIT INFLUENCE KETAMINE-INDUCED HYPNOSIS

Contributions to Chapter IV

This chapter is not published.

Author Contributions

Soll LG – genotyping, designed and performed experiments, performed data analysis, prepared figures, and wrote this chapter.

Guildford MJ, Ngolab J, and Pang X – genotyping

Tapper AR and Gardner PD – designed experiments data analysis and edited chapter.

ABSTRACT

Compounds that produce sedation, immobilization, and analgesia are considered general anesthetics. Ketamine is classified as an NMDA antagonist, but also blocks nicotinic acetylcholine receptors (nAChRs). It is still debated whether concentrations of ketamine necessary to block nAChRs are clinically relevant to the anesthetic actions of ketamine, or only contribute to the drug's side effects. Here we show evidence that nAChRs indeed do modulate the hypnotic effects of ketamine *in vivo*. Using the loss of righting reflex (LORR) assay, we first showed that nicotine, an nAChR agonist, as well as mecamylamine, hexamethonium, and methyllycaconitine (MLA), nAChR antagonists, alter duration but not latency of ketamine-induced LORR. We then compared the ketamine-induced hypnosis of α4 nAChR subunit knock out mice (α4 KO) to wild-type littermates (WT) and found α4 KO had reduced LORR durations, but tolerance to ketamine was unaffected. Additionally, mecamylamine had no effect on ketamine-induced LORR in α4 KO mice. We also found longer LORR durations in α6 KO mice compared with WT. Together, these data suggest that nAChRs play a modulatory role in the hypnotic actions of ketamine.

IV.A. Introduction

Elderly and pediatric surgical patients are the most common recipients of ketamine anesthesia. General anesthetics act as cardiovascular and pulmonary depressants, which can cause life-threatening effects in these patients. Ketamine is unique because it increases blood pressure, heart rate, and cardiac output (White and Ryan, 1996, Alletag et al., 2012). Ketamine's fast induction rate and amnesic and analgesic properties also make this drug a valuable anesthetic for surgical procedures, pre-operative care, pre-administration of other general anesthesia, and pain control (White and Ryan, 1996, Prommer, 2012). Despite ketamine's attributes, widespread use of ketamine has been limited as a result of its negative side effects, such as vomiting and hallucinations (White and Ryan, 1996, Tassonyi et al., 2002, Alletag et al., 2012).

Despite the wide administration of ketamine in the medical field, the mechanism of its actions is still largely unknown. While most research has focused on its main target, N-methyl D-aspartate (NMDA) receptors, *in vitro* systems have established neuronal nicotinic acetylcholine receptors (nAChRs) as additional targets of ketamine (Scheller et al., 1996, White and Ryan, 1996, Furuya et al., 1999, Sasaki et al., 2000). Nicotine-induced currents in PC12 cells, which have endogenous expression of nAChRs, were blocked by ketamine with an IC_{50} of 2.8 μM, which is lower than the clinically relevant concentration range of

ketamine (2-7 μM) (Furuya et al., 1999, Coates and Flood, 2001). Expression of human $\alpha2\beta2$, $\alpha2\beta4$, $\alpha3\beta2$, $\alpha3\beta4$, $\alpha4\beta2$, and $\alpha4\beta4$ in *Xenopus* oocytes found IC_{50} values of ketamine to inhibit ACh-induced currents in nAChRs ranged from 9.5 μM to 92 μM, outside the range of clinical relevance and, based on these values, found that sensitivity to ketamine was greater in $\beta4$* than in $\beta2$* nAChRs and greater in α4* followed by $\alpha3$* and then $\alpha2$* (Yamakura et al., 2000). Coates and Flood found that ketamine was more effective at human $\alpha7$ nAChRs (IC_{50} 20 μM) than human $\alpha4\beta2$ nAChRs (IC_{50} 50 μM) in *Xenopus* oocytes, but once again IC_{50} values were outside the range of clinical relevance (Coates and Flood, 2001). Ketamine blocked ACh-induced currents in *Xenopus* oocytes expressing chick $\alpha4\beta4$ with an IC_{50} value of 0.24 μM, which is much lower than what has been found with expression of human $\alpha4\beta4$ (Flood and Krasowski, 2000). Based on these studies, it is unclear if nAChRs are blocked at clinically relevant doses of ketamine to induce anesthetic effects.

Very little work has examine whether nAChR modulate anesthetic effects of ketamine *in vivo*. Udesky et al. demonstrated that mecamylamine, a non-selective nAChR antagonist, enhanced analgesic effects of ketamine, implicating nAChRs in non-hypnotic properties of ketamine (Udesky et al., 2005). However, it still remains unclear whether concentrations of ketamine necessary to block nAChRs are clinically relevant to the hypnotic actions of ketamine or only

contribute to the drug's analgesic properties and other effects (Furuya et al., 1999, Sasaki et al., 2000, Tassonyi et al., 2002).

To bridge this gap in knowledge, I tested the hypothesis that nAChRs modulate the hypnotic response to ketamine. I discovered that the duration of ketamine-induced loss of righting reflex was altered by nAChR-targeted pharmacological agents and in nAChR subunit knock out (KO) mice, implicating a role for nAChRs in ketamine-induced hypnosis.

IV.B. Materials and Methods

Animals Drug-naïve female C57BL/6 (WT, Jackson laboratories), α4 KO, and α6 KO and their wild-type littermates (WTLM) between the ages of 8 and 14 weeks, were used for loss of righting reflex experiments. The genetic engineering and genotyping protocol for α4 KO and α6 KO mice was previously described (Ross et al., 2000, Champtiaux et al., 2002). Genetically modified mice have been backcrossed to the C57BL/6J background for at least 9 generations. All mice were bred and housed, up to five mice per cage, at the University of Massachusetts Medical School. Mice were kept on a standard 12h light: 12h dark lighting cycle and food and water were accessible *ad libitum*. All experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council (National

Research Council, 1996), as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Drugs Nicotine hydrogen bitartrate (NIC), mecamylamine hydrocloride (MEC), methyllycaconitine citrate salt hydrate (MLA)**,** and hexamethonium (HEX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dihydrobeta-erythrodine hydrobromide (DHβE) was purchased from Tocris Bioscience (Bristol, UK). Ketamine hydrocloride was purchased from Vedco, Inc. (St. Joseph, MO). All drugs were dissolved in 0.9% saline and administered via interperitoneal (i.p.) injection at the indicated doses.

Loss of Righting Reflex The hypnotic effects of ketamine were measured using the loss of righting reflex (LORR) assay. To avoid variation in LORR experiments from stress of injection and handling, mice were given a saline (i.p.) injection once a day for 3 days prior to each experiment. Additionally, on the day of the experiment, mice were habituated to the room for approximately 40 minutes and all experiments were conducted at the same time of day to avoid differential effects of ketamine based on the time of day known as chronopharmacology (Sato et al., 2004). Mice were administered ketamine (100 mg/kg or 200 mg/kg as stated, i.p.) and then the latency to the LORR was measured. The LORR was determined when mice were unable to right

themselves after 5 seconds following supine placement into a V-shaped trough. Mice remained in this position until recovery from the LORR. Mice were considered “recovered” when they could right themselves 3 times in the span of 1 minute. The duration of LORR was calculated by subtracting the time of the initial LORR from the time of recovery.

Loss of righting reflex with nicotine: Mice were pre-administered saline (i.p.) or nicotine (1 mg/kg, i.p., made fresh and titrated to pH 7.2) 5 minutes before ketamine administration. Latency to the LORR and time of recovery were measured and duration of the LORR was calculated as above.

Loss of righting reflex with nAChR antagonists: Mice were pre-administered saline (i.p.) or an nAChR antagonist (3 mg/kg MEC, i.p., 10 mg/kg MLA, 3 mg/kg DHβE, or 1 mg/kg HEX) 15 minutes before ketamine administration. Latency to the LORR and time of recovery were measured and duration of the LORR was calculated as above.

Tolerance to LORR Ketamine (100 mg/kg, i.p.) was administered to α4 KO and WTLM mice and LORR latency and duration were measured. This was repeated for the next 3 consecutive days, thus totaling 4 days. To calculate the percent of baseline, the LORR measurement from each day was divided by the baseline LORR measurement from day 1.

Data Analysis Behavioral data were analyzed with unpaired t-test or one-way ANOVA as indicated using Graphpad Prism 5 software (Graphpad Software, La Jolla, CA, USA). Multiple comparison tests were used to identify differences between groups. Statistical significance was set at $p<0.05$.

IV.C. Results

Mecamylamine (MEC), a non-specific nicotinic acetylcholine receptor (nAChR) antagonist, enhances the analgesic properties of ketamine (Udesky et al., 2005). Therefore, I hypothesized that nAChRs may also modulate the hypnotic effects of ketamine. To test my hypothesis, I pre-administered a nAChR agonist or antagonist before a hypnosis-inducing dose of ketamine (100 mg/kg, i.p.) and then measured latency to LORR and the duration of the LORR (Fig. IV-1). Similar to the findings from Udesky et al, 2005, the latency to the LORR was not significantly altered in the presence of a nAChRs agonist or antagonist (data not shown). Nicotine (NIC) significantly increased duration of LORR in WT mice (Fig. IV-1a) measured by an unpaired t-test ($t=3.575$, $p<0.01$). Complementing these results, MEC (Fig. IV-1b) decreased LORR duration in WT mice ($t=2.666$, $p<0.05$). Selective pharmacological blockade of $\alpha4\beta2$* (* denotes that other subunits may also co-assemble with $\alpha4$ and $\beta2$ subunits in the receptor) nAChRs with DHβE or blocking $\alpha7$ nAChRs with MLA had no significant effect on

ketamine-induced LORR duration (Fig. IV-1c and d, respectively). Interestingly, peripheral blockade of nAChRs with HEX has the opposite effect of MEC and increases sleep duration ($t=2.116$, $p<0.05$). Taken together, this suggests that nAChRs can modulate ketamine-induced LORR duration.

Although ketamine antagonizes α4* nAChR function *in vitro*, the role, if any, *in vivo* is unknown. I hypothesized that α4* nAChRs are likely targets of the hypnotic response to ketamine (Fig. IV-2) (Coates and Flood, 2001). Genetic deletion of the α4 nAChR subunit in mice (α4 KO) altered ketamine-induced LORR duration (Fig. IV-2a) but not latency (data not shown) in a dose-dependent manner. Analysis by two-way ANOVA indicated that there was a significant effect of genotype ($F_{1,29} = 482.6$, $p<0.0001$) and drug treatment ($F_{1,29} = 18.98$, $p=0.0002$) and significant interaction between genotype and drug treatment ($F_{1,29} = 4.487$, $p<0.05$). Further analysis by Tukey's multiple comparisons test indicated that there was a significant difference between drug doses in WTLM ($p<0.0001$) and α4 KO ($p<0.0001$) and between WTLM and α4 KO after 200 mg/kg ketamine ($p<0.01$). Comparing WTLM and α4 KO after 100 mg/kg ketamine revealed significant difference (Tukey, $P<0.05$**)** in this experiment, but a significant effect of genotype was observed at this ketamine dose in other experiments (Fig. IV-2b, IV-2d). To test if α4* nAChRs are also involved in tolerance to ketamine, I measured latency and duration of LORR after consecutive treatments of ketamine for 4 days (Fig. IV-2b). Once again there

was no difference in the latency to LORR between α4 KO and WTLM mice on days 1-4 (data not shown). A two-way ANOVA analysis revealed a significant effect of genotype ($F_{1,51}$= 9.598, p<0.001) and day ($F_{3,51}$= 20.65, p<0.0001) between α4 KO and WTLM in duration of LORR across days 1-4. Differences between the two genotypes in LORR duration were apparent only on day 1 as indicated by a Sidak's multiple comparison test (p<0.01). Figure IV-2c depicts measurements of tolerance to ketamine over 4 days of ketamine administration by analyzing the % difference from baseline LORR (day 1 LORR duration) on each day. There is a significant effect of genotype ($F_{3,51}$= 63.49, p<0.0001) and day ($F_{1,51}$=10.73, p<0.01). Further, a Bonferroni's multiple comparisons test reveals a difference between WTLM and α4 KO on day 4 (p<0.05). Pre-challenging with MEC in α4 KO and WTLM reveal an overall significant effect on drug by two-way ANOVA ($F_{1,21}$ = 5.096, p<0.05). Only a significant difference in drug treatment was apparent in WTLM (Fig. IV-2d). Tukey's multiple comparison test reveal a significant difference between saline- and MEC-treated WTLM (p<0.01), and saline-treated WTLM and saline-treated α4 KO (p<0.01) and MEC-treated WTLM (p<0.01).

To date, a role for α6* nAChRs in the response to anesthetics has not been examined. To test the hypothesis that α6* nAChRs are modulators of ketamine-induced hypnosis I challenged WTLM and α6 knock out (α6 KO) mice with ketamine (100 mg/kg, i.p.) and measured latency and duration of ketamine-

induced hypnosis (Fig. IV-3). An unpaired t-test found a significant difference between WTLM and α6 KO latency to LORR ($t=2.798$, $p<0.05$) and LORR duration ($t=2.576$, $p<0.05$).

IV.D. Discussion

Nicotinic acetylcholine receptors modulate duration of ketamine-induced hypnosis

Previous in vitro studies demonstrated nAChRs are inhibited by ketamine (Flood and Krasowski, 2000, Yamakura et al., 2000). *In vivo* work shows that mecamylamine modulate ketamine's analgesia properties (Udesky et al., 2005). Taken together, I hypothesize that these receptors may also modulate the hypnotic effects of ketamine. Udesky et al. first looked at how pharmacological blockade of nAChRs could change the dose of ketamine required to induce hypnosis, yet failed to see any effect (Udesky et al., 2005). Using a standardized dose of ketamine, I measured both latency to LORR and duration of LORR in response to ketamine plus pharmacological blockade of nAChRs, both measurements that were disregarded in the Udesky et al. study. Latency to the ketamine-induced LORR was unchanged by pharmacological targeting of nAChRs in mice challenged with either a nAChR agonist or antagonist. This suggests that initiation of hypnosis is unaffected by nAChRs. However, nicotine,

mecamylamine, and hexamethonium significantly changed the duration of the LORR, indicating that nAChRs modulate maintenance of the hypnotic state and/or emergence from the hypnotic state.

Most strikingly, my results reveal opposing effects of mecamylamine and hexamethonium on the modulation of ketamine-induced sleep duration (Fig. IV-1b and e, respectively). Both drugs are nAChRs antagonists, but hexamethonium cannot cross the blood brain barrier. Thus, i.p. administration of hexamethonium antagonizes only peripheral nAChRs. My results imply that there are separate roles for nAChRs in the central nervous system (CNS) and the peripheral nervous system (PNS) in modulating the hypnotic actions of ketamine. This may be a reflection of nAChR subtype expression in the PNS versus the CNS. Together this might suggest that modulation of ketamine-induced hypnosis occurs through different nAChR subtypes expressed in multiple pathways mediating ketamine hypnosis.

One caveat to the LORR assay as a measurement of hypnosis is that mice must be capable of righting themselves. It is possible that increased LORR duration by HEX may not be a reflection of peripheral nAChRs involvement in hypnosis but rather a secondary affect. Hypnosis and immobility are controlled by the CNS, whereas muscle relaxation is an effect of the PNS (Tassonyi et al., 2002, Rudolph and Antkowiak, 2004). Two subtypes of nAChRs, heteromeric $\alpha 3\beta 4$*

receptors and homomeric α7 nAChRs, have already been established in ketamine's actions in the PNS (Tassonyi et al., 2002). Therefore it is likely that ketamine blocks peripheral α3β2* and α7 nAChRs, leading to an increase in muscle relaxation and other effects that impedes righting ability, resulting in increased LORR duration after ketamine plus HEX.

Homomeric α7 nAChRs do not modulate ketamine-induced hypnosis.

Sub-anesthetic concentrations of ketamine and its metabolites inhibit α7 nAChRs *in vitro* (Coates and Flood, 2001, Tassonyi et al., 2002, Moaddel et al., 2013). As a result, I wanted to test the hypothesis that these receptors were also involved in the hypnotic response to ketamine. I found that MLA, an agonist selective for α7 nAChRs, had no affect on ketamine-induced hypnosis (Fig. IV-1d). One caveat is that I only used one dose of MLA and ketamine. It is possible that MLA may have an effect with a higher dose of ketamine or a larger dose of MLA may be used. The dose of MLA used in this study (10 mg/kg) is higher than the dose required to induce a nicotine withdrawal syndrome in mice (7.5 mg/kg), therefore 10 mg/kg MLA should be sufficient to induce behavioral differences with ketamine if α7 nAChR are involved (Damaj et al., 2003). From experiments performed, I conclude that α7 nAChRs modulate non-hypnotic effects of ketamine.

Opposing modulation of ketamine-induced hypnosis by α4 and α6* nAChRs*

Ketamine can inhibit α4* nAChRs in oocytes (Flood and Krasowski, 2000, Yamakura et al., 2000, Coates and Flood, 2001). For that reason, I hypothesized that α4* nAChRs modulate the hypnotic effects of ketamine. Consistent with my pharmacological experiments, latency to LORR was not changed by genetic deletion of α4 nAChRs, however the duration of LORR was decreased at each of the two doses tested in α4 KO mice (Fig. IV-2a). It is surprising that LORR experiments indicated that hypnotic duration was altered in α4 KO animals, yet pharmacological blockade of α4β2* nAChRs using DHβE had no effect (Fig. IV-1c). One possibility is that α4 KO mice may metabolize ketamine at a different rate than WTLM and this possibility should be examine in future studies. It is also possible that differences in LORR duration in α4 KO mice and not by DHβE may suggest α4(non-β2)* nAChRs modulate ketamine-induced hypnosis because DHβE is more selective for α4β2* nAChR. A likely subtype is the α4β4* nAChR, which has a higher sensitivity to ketamine than α4β2* nAChRs and is expressed in brain regions associated with anesthetic responses, such as the habenula and interpeduncular nucleus (IPN), and cerebellum (Flood and Krasowski, 2000, Yamakura et al., 2000, Tassonyi et al., 2002).

The rapid tolerance to the hypnotic effects of ketamine has been reported to be mediated by the CNS rather than an increase in rate of drug metabolism (White

and Ryan, 1996). Using mice expressing hypersensitive α4* nAChRs, Tapper et al. found that α4* nAChRs were sufficient for tolerance to nicotine-induced hypothermia (Tapper et al., 2004). Furthermore, α4 KO mice failed to show tolerance to nicotine-induced hyperactivity (Cahir et al., 2011). Together, this shows a necessity and sufficiency of α4* nAChRs to mediate tolerance to nicotine. I tested the role of α4* nAchRs in tolerance to ketamine by measuring ketamine-induced LORR over 4 consecutive days (Fig. IV-2b and c). Decreased duration to ketamine hypnosis in α4 KO mice was only significant the first day of administration and completely vanished by day 3**.** To measure differences in tolerance, I compared the percent of day 2-4 duration of LORR to that of day 1 (baseline). In WTLM and α4 KO, there is a rapid tolerance to ketamine indicated by the decreased LORR duration between day 1 and 2 and by the decreased percent of baseline on day 2 (Figure IV-2b and c). The drop in percent of baseline on day 2 did not decrease further in either α4 KO or WTLM, suggesting there was no further tolerance after day 2 in these two populations. Additionally, there was no difference between the percent of baseline between α4 KO and WTLM, except on day 4. Overall, I conclude that α4* nAChRs do not significantly modulate tolerance to ketamine-induced hypnosis. Since tolerance to ketamine is not affected by α4* nAChRs, this also further supports the idea that ketamine metabolism is not responsible in acute LORR differences between genotypes.

The α6 nAChR has only recently become of interest and a role for these receptors has yet to be evaluated in general anesthesia (Flood and Role, 1998, Tassonyi et al., 2002, Changeux, 2006, Yang et al., 2009). It is a potential candidate involved in the anesthetic response because of its expression in brain regions implicated in anesthetic actions, such as the thalamus, IPN, and habenula (Herkenham, 1981, Champtiaux et al., 2002, Franks, 2008). I found a significant decrease in latency to LORR in α6 KO mice. Surprisingly, this was the only receptor I identified to influence latency to LORR, modulating induction of hypnosis. Additionally I measured a significant increase in duration of hypnosis indicating modulation of either maintenance or emergence from hypnosis.

The opposing modulation of duration of LORR by α4* and α6* nAChRs is intriguing and may be a reflection of the differential expression pattern of these two receptor subtypes. α4* nAChRs are widely expressed in a variety of different neuronal subtypes (e.g., dopaminergic and GABAergic neurons) throughout the CNS and PNS. On the other hand, α6* nAChRs are mainly restricted to catecholaminergic neurons in the CNS, specifically in the midbrain, ventral tegmental area, and substantia nigra, and also expressed in the thalamus and IPN (most likely on neuron terminals) (Le Novere et al., 1996, Klink et al., 2001, Azam et al., 2002, Yang et al., 2009). The opposing results obtained by blocking α4* or α6* nAChRs may imply a mechanism, based on their expression patterns,

in which these two nAChR subtypes regulate different processes within the same pathway or modulate two different pathways.

Some theorize that there is a shared circuitry mediating hypnosis amongst all anesthetics (Rudolph and Antkowiak, 2004, Alkire, 2008). Under that assumption, it is no surprise that nAChRs play a role in modulating the effects of ketamine since they have been implicated in modulating hypnotic actions of other general anesthetics such as inhalation anesthetics (Alkire et al., 2007, Yan et al., 2009). One study reported shorter sleep durations after intracerebroventricular infusion of nicotine in mice anaesthetized with emulsified enflurane, isoflurane, or sevoflurane (Yan et al., 2009). Additionally, mecamylamine had no effect on induction of hypnosis by sevoflurane, however intrathalamic infusion of nicotine awakened mice out of the sevoflurane-induced sleep (Alkire et al., 2007). Both studies complement my data and together suggest nAChRs modulate maintenance and/or emergence from drug-induced hypnosis. Moreover, the study by Alkire et al, implicates thalamic nAChRs in mediating arousal (Rudolph and Antkowiak, 2004, Alkire et al., 2007). The thalamic network has already been linked to arousal from anesthesia, including ketamine (Rudolph and Antkowiak, 2004, Alkire, 2008, Franks, 2008, Hwang et al., 2012). α4* nAChRs are likely targets. Although widely expressed, these receptors are densely populated in the thalamus. Additionally, my data showing α4 KO mice have

decreased ketamine-induced sleep duration support a role for α4* nAChRs in the maintenance and/or emergence from a ketamine-induced hypnotic state.

This work provides the first evidence of nAChR-mediated modulation of ketamine-induced hypnosis. Further experiments need to be carried out to elucidate how α4* and other nAChRs (e.g., α6* nAChRs) modulate different functions of hypnosis. Such knowledge will direct the development of drugs to supplement ketamine improving safety and efficacy of anesthesia.

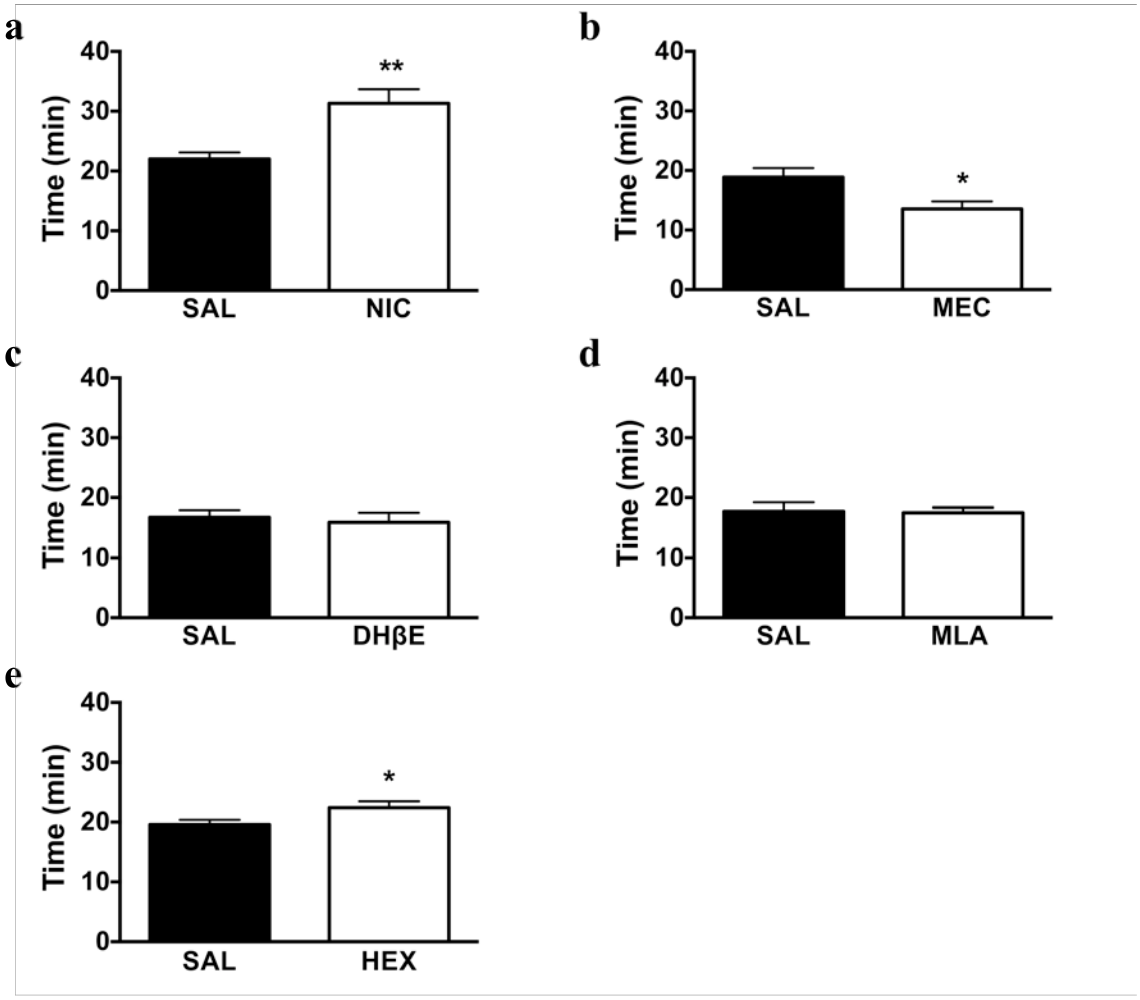

Figure IV-1. Pharmacological modulation of nAChRs alters duration of ketamine anesthesia in WT mice

Duration of ketamine-induced LORR in WT mice. a) Administration of saline (i.p. n=8) or nicotine (1 mg/kg nic, i.p, n=8) 5 minutes prior to ketamine (100 mg/kg, i.p.) b-e) Saline or drug was administered 15 minutes before ketamine (100mg/kg, i.p.) b) Saline (i.p. n=7) and mecamylamine (3 mg/kg, i.p. n=14), c) Saline (i.p., n=8) and DHβE (3 mg/kg, i.p., n=8), d) Saline (i.p., n=14) and MLA (10 mg/kg, i.p., n=14) e) Saline (i.p., n=15) and hexamethonium (1 mg/kg, i.p., n=14) *p <0.05 and **p<0.01 in unpaired t-test.

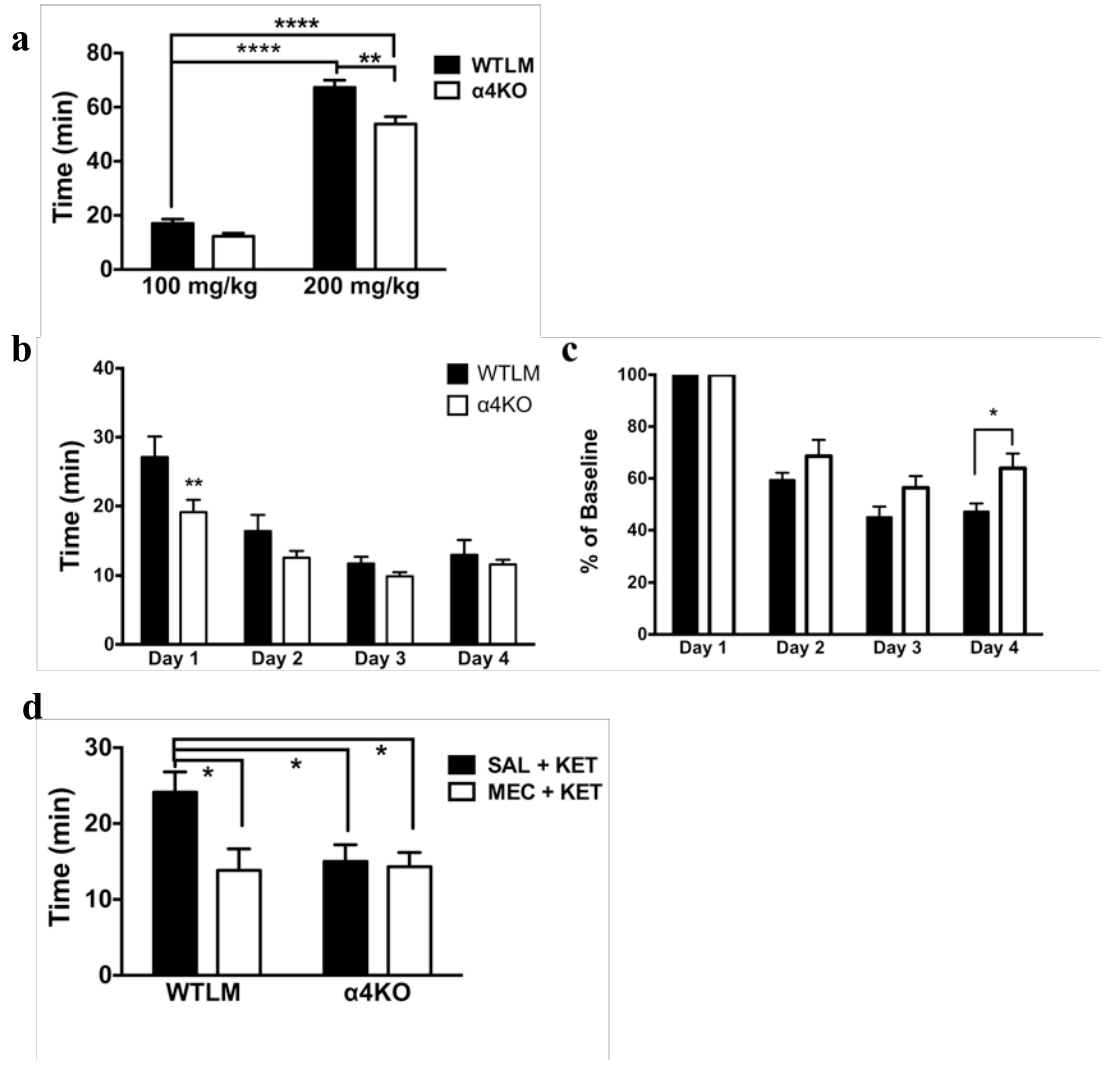

Figure IV-2. α4 KO have decreased ketamine-induced sleep

Duration of ketamine induced sleep (a-d). a) α4 KO and WTLM response to 100 mg/kg (WTLM = 9, α4 KO= 10) and 200 mg/kg (WTLM, n= 5, α4 KO, n=9) ketamine (i.p.) b) Ketamine (100 mg/kg, i.p.) was administered for 4 consecutive days and duration of LORR was measured in WT (n=7) and α4 KO (n=8). c) The percent of baseline duration of LORR. d) Saline (i.p.) (WTLM, n=7, α4 KO, n =7) or mecamylamine (3 mg/kg, i.p.)(WTLM, n=5, α4KO, n=6) administration 15 minutes before ketamine (100 mg/kg, i.p.) *p<0.05, **p<0.01, ****p<0.0001 in a two-way ANOVA with either a Tukey's (a and d) or Sidak's (b) or Bonferroni's (c) multiple comparison's test.

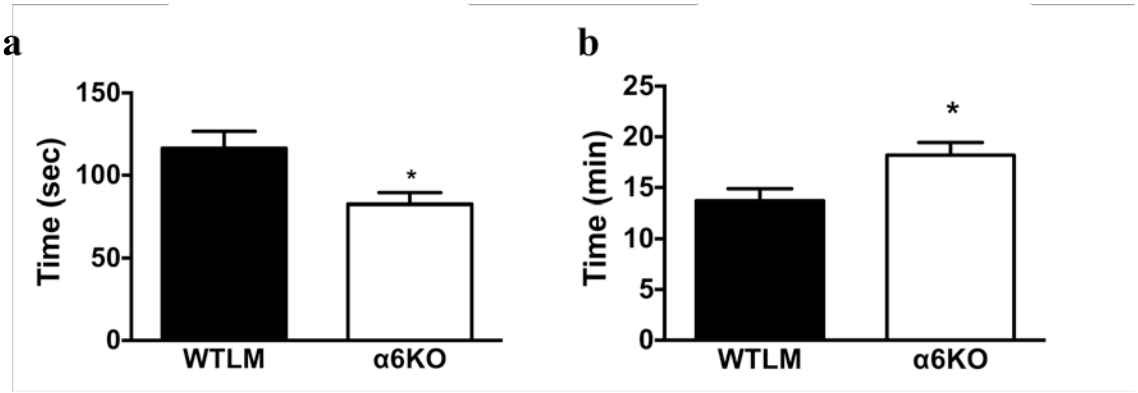

Figure IV-3. Ketamine induces longer LORR in α6 KO mice
WTLM (n=7) and α6 KO (n=8) mice were administered ketamine (100 mg/kg, i.p.) and a) latency to LORR and b) duration of ketamine-induced LORR *p<0.05 in a unpaired t-test.

CHAPTER V.

DISCUSSION

V.A. α4(non-α6)* nAChRs are necessary for modulating motor output

Chapters II and III present evidence for involvement of the α4* nAChR in movement. The foundation of this research is based on evidence which indicates that striatal dopamine release is modulated by α4* nAChRs, however manipulation of these receptors through pharmacological antagonism or knock-out nAChR subunit mouse models had yet to establish a significant role for these receptors in motor output (Ross et al., 2000, Salminen et al., 2004, Jackson et al., 2009a, Threlfell et al., 2012).

In order to demonstrate that α4* nAChRs play a role in spontaneous motor behavior, we utilized our knock-in mouse model which expresses a point mutation in the α4 subunit rendering receptors 50-fold more sensitive to agonists than a wild-type receptor (Tapper et al., 2004). We hypothesized that these mice are more sensitive to basal levels of ACh; in these mice, blocking hypersensitive receptors would result in observable changes in motor function that are not apparent in WT mice. Blocking hypersensitive receptors with the α4β2* nAChR selective antagonist DHβE in both homozygous (Chapter II) and heterozygous (Chapter III) Leu9'Ala mice induced a motor phenotype characterized by hypolocomotion, akinesia, catalepsy, tremor, hind-paw clasping, and abnormal posture. These symptoms (except hindlimb clasping) are characteristic of those

in Parkinson's disease, which arise from low striatal DA levels as a result of the degeneration of SNpc DAergic neurons (Martin et al., 2011a). We therefore hypothesized that striatal DA levels in the Leu9′Ala mice challenged with DHβE would also be reduced. Pharmacological targeting of the DAergic system through amphetamine, D_1R agonism or D_2R antagonism, could rescue the DHβE-induced hypolocomotor activity in Leu9′Ala homozygous mice. These data indicated a role for DA in the phenotype.

C-Fos gene expression data in Chapter II indicated activation of neurons in the striatum and substantia nigra pars reticulata was present in Leu9'Ala homozygous but not WT mice challenged with DHβE, which is consistent with activation of the indirect DA pathway, a circuit that regulates suppression of movement and becomes overly active in PD patients (Gerfen and Surmeier, 2011). I hypothesize that c-Fos expression in the striatum is present in D_2R expressing MSNs. This assumption is based on evidence that expression of c-Fos in the striatum was in non-cholinergic neurons and over-activation of the indirect pathway can lead to motor deficits similar to symptoms observed in this model. Identification of the type of c-Fos expressing neuron would be useful to test this hypothesis. To distinguish between D_1R and D_2R expressing neurons, neuropeptides that are associated with their respective DA receptors, such as dynorphin (for D_1R MSNs) and enkephalin (for D_2R MSNs), can be used as markers (Steiner and Gerfen, 1998, Reiner et al., 1999). If c-Fos is not

expressed in these neurons, then it is possible that GABAergic interneurons may be involved. To identify different subtypes of GABAergic interneurons, markers for somatostatin, parvalbumin/calretinin, or neuropeptide Y/nitric oxide synthase may be used (Tepper et al., 2010).

Although c-Fos was apparent in the striatum of DHβE-challenged Leu9'Ala mice, c-Fos expression was not as extensive as I predicted since DA antagonism by haloperidol induces c-Fos expression in 100-200 per slice in the striatum in mice (Patel et al., 1998). I looked for c-Fos activation 180 minutes after the initial DHβE injection to capture c-Fos activation when the DHβE-induced phenotype was most robust. It is possible that the phenotype may occur as a consequence of neuronal activation at a much earlier time point, therefore we are not measuring at the optimal time point necessary to detect the peak of c-Fos activation. A time-course of c-Fos activation would be needed to identify the peak of c-Fos activation, which may occur earlier or later than the time point captured in the c-Fos experiment in Chapter II. Furthermore, c-Fos is a marker for neuronal activation. We are likely measuring neuronal disinhibition, however, since DHβE is an antagonist. This is important because we cannot detect all the regions that are directly affected by DHβE since inhibition cannot be detected by c-Fos. Therefore, it is likely that other brain regions may also play a role in the motor phenotype induced by DHβE.

We were interested in further investigating whether α4α6β2* nAChRs modulated the phenotype induced by DHβE in Leu9′Ala mice in Chapter II. The α4α6β2* nAChR subtype is expressed on DAergic terminals and has the highest affinity for nicotine (Salminen et al., 2004, Grady et al., 2007, Salminen et al., 2007). This subtype also modulated hyperactivity exhibited in a gain-of-function α6* nAChR mouse model (Drenan et al., 2010). ACh-evoked DA release in synaptosomes from Leu9′Ala mice revealed that hypersensitivity to ACh and nicotine was independent of the α6 subunit. Genetic deletion of the α6 nAChR subunit in Leu9′Ala mice had no effect on DHβE-induced hypolocomotion, which reveals that α4(non-α6)* nAChRs modulate a motor deficit induced by DHβE in Leu9′Ala mice. Together these models present evidence that α4(non-α6)* and α4α6* nAChRs play separate roles in mediating DA release and motor output.

The synaptosome data presented in Chapter II also reveal other interesting information about the Leu9'Ala mice. The higher sensitivity of the Leu9'Ala mice to nicotine and ACh was not detected in the synapotosomes. This may be accounted for by the fact that we were measuring total DA released from synpatosomes. Other nAChRs expressed in the terminal and in post-synaptic fractions included in the synpatosome affect total DA release. Additionally, downregulation of Leu9'Ala α4* nAChRs in Leu9'Ala mice may compensate for hypersensitivity to ACh and nicotine, which may be reflected in a lack of sensitivity to ACh and nicotine measured by total DA release in synaptosomes.

This is supported by our synaptosome data that revealed a downregulation of α4* nAChRs in Leu9'Ala mice indicated by a decreased R_{max} value in the α-CtxMII-resistant component for nicotine and ACh in Leu9'Ala mice compared to WT (Table II-2). Additionally, R_{max} value in the α-CtxMII-sensitive component was increased in Leu9'Ala mice suggesting that the α6* nAChRs were upregulated (Table II-2). Together, this suggests that there is compensation by the number of expressed receptor subtypes in Leu9'Ala mice.

There are still many questions surrounding the mechanism of the gain-of-function mutation in the Leu9′Ala mouse. This mutation increases sensitivity of α4* nAChRs to agonists by 50-fold via changing the gating properties of the receptor, not by altering the binding sites for agonists or antagonists (Tapper et al., 2004). I predict that basal levels of ACh have a larger effect on hypersensitive α4* nAChRs than other nAChR subtypes in the striatum. If this were true, then higher striatal DA levels would be expected to result in Leu9′Ala mice exhibiting basal hyperactivity. Since hyperactivity was not apparent in these mice, it suggests that compensation occurs to counteract the heightened response to ACh. Basal levels of DA in the striatum do not differ between Leu9′Ala and WT mice and neither do their responses to AMPH and to D_1R agonism and D_2R antagonism (Zhao-Shea et al., 2010, Soll et al., 2013). This suggests that compensation does not occur in the amount of DA release or mechanisms downstream in DA signaling. After reviewing these data, I hypothesize that

compensation occurs in cholinergic signaling or the number of Leu9′Ala receptors available for activation. There are two possible ways that receptors could be affected: decreasing number of Leu9′Ala receptors expressed or larger numbers of Leu9′Ala receptors exists in a desensitized state, meaning they are already bound by agonist and in an inactive state. In both scenarios a smaller number of receptors would be available to modulate DA release, therefore DA release would be comparable to WT levels. Then, with a smaller subset of active Leu9′Ala receptors, antagonism would have a greater effect. Synaptosome data (discussed earlier) suggests that there is a downregulation of α4* nAChRs in the Leu9′Ala mice which may account for normal DA striatal levels and heightened response to blocking these receptors with DHβE. In the latter case, if fewer Leu9′Ala α4* nAChRs are expressed, blocking these receptors would potentially lead to a greater response as a result of a greater dependency on the expressed receptors to provide normal DA release.

Although the Leu9′Ala mutation in the α4 subunit does not occur naturally, the Leu9′Ala model is a tool that can be used to study the role of endogenous α4* nAChRs in motor output. In a normally functioning DA system in the basal ganglia, α4* nAChRs may not have a large effect on motor function since DA levels required for motor function are relatively stable: only major decreases lead to motor deficits. It is likely that other receptors may compensate for small, temporary DA losses. In a condition where there are consistently low DA levels,

such as in PD, these α4* nAChRs may have a larger impact. In a recent study, an agonist for β2* nAChRs, TC-8831, improved motor coordination after 6-hydroxydopamine lesioning in mice, supporting nAChRs as a promising class of targets for the therapeutic treatment of PD (Kucinski et al., 2013). Currently the most effective treatment for PD is dopamine replacement using L-DOPA, however tolerance to the treatment reduces its effectiveness over time (Quik et al., 2011b). My data highlights the importance of α4* nAChR in motor function and therefore suggests that targeting α4* nAChRs using nAChR allosteric modulators or agonists in order to promote DA release may be effective treatments for PD or may also be used in combination with lower doses of L-dopa to help improve the duration of L-DOPA effectiveness in PD treatment (Quik et al., 2012).

Future Directions

1. Are striatal DA levels decreased in Leu9'Ala mice challenged with DHβE?

Hypolocomotion, rigidity, postural abnormalties, and tremors are symptoms are Parkinson's disease resulting from low dopamine levels in the St caused from the progressive degeneration of DAergic neurons in the SNpc (Martin et al., 2011a). Leu9′Ala mice challenged with DHβE exhibit a similar motor phenotype. Therefore, I propose that DHβE-induced motor symptoms in Leu9′Ala mice are

caused by a depletion of striatal DA as a consequence of blocking α4* nAChRs on SNpc DAergic neurons. Pharmacologically targeting of the DAergic system to block the phenotype provides supportive data for this hypothesis, however it is necessary to measure DA concentrations after DHβE challenge in Leu9′Ala and WT mice to confirm my hypothesis. Striatal DA concentrations should be measured every half an hour over the span of 4 hours (the average time that the phenotype lasts) after DHβE administration. Since we are interested in measuring the amount of DA released into the striatum, the best ways to measure this would be to use either microdialysis or fast-scan cyclic voltammetry (FSCV). Both techniques can be performed *in vivo* and will provide the temporal resolution necessary to measure DA levels in the striatum at multiple time points after DHβE administration. Microdialysis is better suited for our interests because were are interested in time resolution in minutes to hours and FSCV is better used for sub-second time resolution (Robinson et al., 2003). This may provide interesting information correlating behavioral symptoms onset with specific decreases in DA concentrations.

2. Is nicotine neuroprotective or is upregulation of α4* nAChRs masking motor symptoms after DAergic lesion?

Smokers have a lower incidence of PD, suggesting that nicotine has neuroprotective effects (Thacker et al., 2007, Chen et al., 2010). The

mechanisms for nicotine's neuroprotective effects remain to be completely elucidated. It is possible that upregulation of nAChRs as a result of smoking may contribute to masking symptoms of neurodegeneration. Upregulation of nAChRs occurs in the striatum after a mild MPTP-induced lesion in a pre-symptomatic PD mouse model (Kryukova et al., 2013). It is theorized that the upregulation of nAChRs increases DA neuron firing as a compensatory mechanisms to mask mild to moderate loss of DAergic neurons**.** Potential nAChR candidates are $\alpha4\beta2$* nAChRs because they are preferentially upregulated after chronic nicotine (Gotti et al., 2009, Quik and Wonnacott, 2011). Additionally, $\alpha4\alpha6\beta2$* nAChRs expressed on DA terminals in the striatum are vulnerable to drug-induced lesion, suggesting they are highly affected by lesioning (Bordia et al., 2007). Neuroprotective effects of nicotine are also inhibited in $\alpha4$ KO mice and DHβE inhibits nicotine protection against rotenone-induced DAergic degeneration (Ryan et al., 2001, Takeuchi et al., 2009). Collectively this provides evidence for the necessity of $\alpha4\beta2$* nAChRs in nicotine's neuroprotective effects against lesioning. I hypothesize that $\alpha4$* nAChRs are also sufficient for neuroprotective effects of nicotine in response to drug-induced neurodegeneration. To test this hypothesis, Leu9′Ala mice could be chronically treated with low concentrations of nicotine, therefore selectively activating only hypersensitive receptors, before a 6-hydroxydopamine-induced lesion. If $\alpha4$* nAChRs are indeed sufficient for neuroprotection, then nicotine-treated Leu9′Ala mice would exhibit a reduction in 6-hydroxydopamine lesioning

compared to nicotine-treated WT mice. The power of this model is that nicotine levels used to chronically treat Leu9′Ala mice would only affect the hypersensitive receptors. It is also possible that the hypersensitive mutation would be enough for neuroprotective effects so lesions in control animals (drug-naïve Leu9′Ala and WT mice) are necessary to identify if any differences in the absence of nicotine treatment. This experiment would rule out modulation by other nAChR subtypes and provide further evidence that α4* nAChRs are therapeutic targets for PD.

3. Where is DHβE (specifically) acting to induced motor symptoms?

The robust nature of the DHβE-induced motor phenotype in Leu9′Ala mice could be a consequence of global Leu9′Ala α4* nAChR antagonism. Expression of α4* nAChRs is not limited to the basal ganglia, but expressed in other motor-related areas, particularly the spinal cord (Wada et al., 1989). It is possible that localized antagonism of α4* nAChRs in the striatum of Leu9′Ala mice or localized expression of Leu9′Ala α4* nAChRs in a WT animal would not necessarily replicate the phenotype, but rather mimic certain aspects such as hypolocomotor activity. One experiment to assess which phenotypes are associated with the striatum is to locally knock-down the α4 subunit in Leu9′Ala mice in the SNpc and then challenge mice with DHβE and test for motor deficitsIt is possible that since α4* nAChRs are expressed in the spinal cord, postural abnormalities may occur as well as related motor deficits. The reverse of this experiment can also be

accomplished where Leu9'Ala α4* nAChRs can be expressed in SNpc DAergic neurons using a viral mediated re-expression system in DAT-Cre mice. Challeneging mice containing hypersensitive α4* nAChRs soley in the SNpc with DHβE would test the sufficiency of α4* nAChRs in the SNpc to induce specific motor deficits.

V.B. A model for α4* nAChRs in DA-related behaviors

In Chapter III, I expanded my investigation of the effects of DHβE in Leu9′Ala to test the hypothesis that blockade of Leu9′Ala α4* nAChRs affects other DA pathways. Decreased DA levels in the nucleus accumbens (NAc), a brain region within the reward circuitry, accompany nicotine withdrawal symptoms (Hildebrand et al., 1998, Liu and Jin, 2004). I measured DHβE-induced hypolocomotion and conditioned place aversion in Leu9′Ala mice, both common assays to test for a withdrawal state. Hypolocomotor activity and a CPA response in Leu9'Ala heterozygous mice challenged with DHβE suggests that blocking hypersensitive α4* nAChRs lowered DA levels in the NAc. I hypothesize that Leu9′Ala mice may have an intrinsic dependency on ACh due to their hypersensitivity, enabling the manifestation of a withdrawal state in nicotine-naïve Leu9′Ala heterozygous mice. Normally withdrawal would require chronic nicotine treatment followed by its discontinuation to elicit a response in WT mice, however in the Leu9′Ala

model simply blocking hypersensitive α4* nAChRs is sufficient to induce the withdrawal-like state without chronic nicotine treatment.

Future Directions

1. Are accumbal DA concentrations decreased by DHβE treatment in Leu9′Ala mice?

Demonstrating lowered accumbal DA concentrations after DHβE challenge in Leu9′Ala mice would verify my hypothesis that DA decreases in the reward circuit. It would additionally be likely that DA decreases would be measured in other DA signaling pathways that are modulated by α4* nAChRs. Along with microdialysis data from the striatum, DA measurements in the NAc would characterize the reduction in DA over time, which could then be correlated to onset of hypolocomotion and CPA. This could provide a unique perspective on DA and behavioral onset.

2. Do Leu9’Ala challenged with DHβE exhibit other affective symptoms?

Leu9’Ala mice condition a place aversion to DHβE and exhibit hypolocomotor activity, which are associated with an affective nicotine withdrawal state. However, the affective withdrawal state is characterized by a variety of behaviors

including anxiety and reduced reward. Therefore other affective nicotine withdrawal associated behaviors (see Tables I-1 and I-2) need to be tested to assess if a true nicotine withdrawal-like syndrome is being produced (as expected if blocking α4* nAChRs in Leu9′Ala mice leads to low DA concentrations in the NAc).

Interestingly, PD patients also suffer from non-motor symptoms including anxiety and depression (Shiba et al., 2000, Ishihara and Brayne, 2006, Poletti et al., 2012). These non-motor symptoms are thought to pre-date the onset of motor symptoms (Ishihara and Brayne, 2006, Jacob et al., 2010). The onset of the withdrawal-like state and motor symptoms induced by DHβE in Leu9′Ala mice mimics the onset of non-motor and motor PD symptoms. Varenicline, an FDA-approved α4* nAChR partial agonist, is used for smoking cessation and relieves affective withdrawal symptoms (Gonzales et al., 2006, Jorenby et al., 2006, West et al., 2008). Since PD patients also suffer from a similar affective state, varenicline or other α4* nAChR-targeted drugs may not just treat motor symptoms but additionally may alleviate affective symptoms and possibly function as a neuroprotective agent.

2. Are α4* nAChRs sufficient to induce affective symptoms in the VTA?

I show that α4* nAChRs are important to anti-reward associated behaviors such as condition place aversion and hypolocomotor activity. In mice chronically treated with nicotine, a localized infusion of MEC into the VTA not only decreased locomotor activity and conditioned a place aversion, but it also reduced NAc DA levels by 25% (Hildebrand et al., 1999). A direct infusion of DHβE into the VTA of Leu9'Ala mice can be used to test the hypothesis that α4* nAChRs specifically in the VTA are sufficient to conditioned place aversion and hypolocomotor activity response. If this is not sufficient, other regions that express α4* nAChR and have been associated with affective symptoms, such as the IPN and habenula may also be tested. This approach could provide evidence that α4* nAChRs in the VTA are important in withdrawal.

V.C. nAChR modulation of the hypnotic state induced by anesthetic drugs

Chapter IV and Appendix I investigate the role of nAChRs in the hypnotic effects of two drugs, ketamine and ethanol. Anesthetic drugs are hypothesized to use the same pathways involved in the sleep/wake cycle in order to induce unconciousness (Rudolph and Antkowiak, 2004). Interestingly, many brain regions associated with consciousness receive cholinergic signaling, providing a link to nAChR involvement in sleep and wakefulness and anesthesia (Perry et al.,

1999). Lesion of the pedunculopontine tegmental (PPTg) nucleus, a key source of ACh for the basal ganglia, disrupts sleep/wake transitions and affects cortical activation during periods of wake, NREM, and REM states, however, it is undetermined if these lesions affect anesthesia (Petrovic et al., 2013). Functional differences in brain activity by anesthetic drugs occur in brain regions associated with sleep and wakefulness. For example, in anesthetized rats ACh turnover is decreased throughout the brain by halothane and in the caudate nucleus and hippocampus with ketamine (Ngai et al., 1978). Ketamine-induced increases in regional cerebral blood flow in the anterior cingulate cortex and increases in the orbital frontal region in healthy human subjects were reduced with nicotine exposure (Rowland et al., 2010). These data provide further evidence that ACh influences the sleep/wake cycle and anesthesia.

Nicotine also modulates the hypnotic effects of volatile anesthetics, establishing a role for nAChRs in the hypnotic properties of anesthetics (Yan et al., 2009). Based on this evidence and previous studies that show nAChR are inhibited by ketamine (Flood and Krasowski, 2000, Yamakura et al., 2000, Coates and Flood, 2001), I investigated a role for nAChR modulation of the hypnotic effects of ketamine. Nicotine, mecamylamine, and hexamethonium did not affect initiation of ketamine-induced hypnosis, but all these drugs modulated duration of ketamine-induced hypnosis. I also investigated different subunits that modulate this response. MLA had no effect, suggesting α7 nAChRs do not modulate

hypnotic responses. Although DHβE also did not have an effect on duration of hypnosis, α4 KO mice displayed reduced ketamine-induced LORR durations, suggesting that hypnosis was modulated by α4(non-β2)* nAChRs. In addition, α6 KO mice exhibited a shorter latency and a longer duration of ketamine-induced hypnosis. Taken together, my data demonstrate that α4* and α6* nAChRs play a role in ketamine-induced hypnosis.

Future Directions

1. Where do α4* and α6* nAChRs modulate the hypnotic response to ketamine?

By comparing ketamine-induced neuronal activation, using c-Fos as a marker, in WT and α4 KO and α6 KO mice, I would be able to identify brain regions that are activated by anesthetic doses of ketamine and ethanol. Differences in c-Fos expression between mouse strains will most likely be moderate since all mouse strains exhibit drug-induced hypnosis. It is important to note that c-Fos expression is not immediate and therefore c-Fos activity will not provide information about brain regions are involved in the different states of hypnosis: induction versus maintenance, and emergence. Recording EEG activity or functional magnetic resonance imaging would offer better time resolution. Further LORR studies using a viral re-expression of nAChR subtypes into specific brain regions or direct infusion of ketamine and ethanol or nAChR

targeting drugs (in combination with an i.p. injection of ketamine or ethanol) into specific brain region would further specify which regions are important in the hypnotic effects of these drugs. A thalamic infusion of nicotine awakened rats out of a sevoflurane-induced sleep (Alkire et al., 2007). This study highlights the thalamus as a potential region in which nAChRs may modulate hypnotic effects of anesthetics.

2. Are nAChRs involved in the hypnotic effects of other anesthetic drugs?

Since nAChRs are inhibited by other anesthetic drugs *in vitro*, it is likely nAChRs also modulate the hypnotic effects of these drugs *in vivo*. In order to test this, α4 and α6 nAChR KO models would be utilized to test the hypnotic effects, using the LORR assay, of other anesthetic drugs such as pentobarbital. Comparing c-Fos in KO models and WT mice may also provide further information about brain regions that are involved. This may then be combined with ketamine c-Fos data to identify brain region in which nAChRs modulate hypnotic effects of these drugs.

V.D. Conclusion

My thesis explores how DHβE treatment of Leu9′Ala mice provides a model to study the role of α4* nAChRs in motor behaviors and an aversive state

associated with DA loss. I also provide evidence that α4* and α6* nAChRs influence the hypnotic responses to ketamine. Taken together, these data demonstrate the diverse roles of nAChRs in anesthesia-induced hypnosis and DA-related behaviors and point to the potential of nAChRs as attractive targets in neurodegeneration and treatment of PD symptoms.

APPENDIX I.

THE α4 SUBUNIT OF NICOTINIC ACETYLCHOLINE RECEPTORS IS INVOLVED IN TOLERANCE TO HIGH ETHANOL CONCENTRATIONS

Contributions to Appendix I

This chapter is not published.

Author Contributions

Soll LG – genotyping, designed and performed experiments, performed data analysis, prepared figures, and wrote this chapter.

Guildford MJ, Ngolab J, and Pang X – genotyping

Tapper AR and Gardner PD – designed experiments data analysis and edited chapter.

ABSTRACT

According to the World Health Organization, alcohol contributes to approximately 2.5 million deaths annually. The molecular mechanisms of alcohol's actions are widespread, but it is becoming more evident that neuronal nicotinic acetylcholine receptors (nAChRs) play a large role in alcohol's rewarding properties and other behaviors associated with alcohol addition. Many nAChR subunits, including α3, α5, α6, α7, and β2 have been implicated in alcohol's hypnotic effects, however, a role for α4 has yet to be established. Here, I explored the hypnotic effects of high doses of alcohol by testing the loss of righting reflex (LORR) in α4 KO mice. I found that α4 KO mice do not differ in latency or duration of the LORR to an acute dose of ethanol, but α4 KO are more resistant to tolerance to the duration of ethanol-induced hypnosis after repeated exposures to ethanol. These data suggest that α4* nAChRs play a role in tolerance to high doses of ethanol.

AI.A. Introduction

Excessive drinking contributes to 79,000 deaths and accounts for $223.5 billion dollars in medical costs a year in the US alone (Naimi, 2011). It is estimated that about 70-75% of alcohol-dependent people also smoke, even though smoking rates over the past decade have dropped in the US (Bobo and Husten, 2000). Given that there is a strong association between smoking and drinking, it has been suggested that nicotinic acetylcholine receptors (nAChRs), which modulate nicotine dependence, may also play a role in ethanol-related behaviors and could be promising therapeutic targets for alcoholism (Davis and de Fiebre, 2006, Hendrickson et al., 2013).

Ethanol elevates dopamine (DA) release into the nucleus accumbens (NAc) from DAergic neurons originating in the ventral tegmental area (VTA), which is characteristic of all rewarding drugs (Hendrickson et al., 2013). Increases in DA concentrations by ethanol can be inhibited by mecamylamine, a non-selective nAChR antagonist, administered systemically or by a direct infusion into the VTA in rodents (Blomqvist et al., 1997, Larsson et al., 2002). Mecamylamine also reduces ethanol self-administration, which suggests nAChRs modulate ethanol's rewarding effects (Ericson et al., 1998, Hendrickson et al., 2009). More recent studies have identified that α4* nAChRs modulate ethanol reward and drinking (Kuzmin et al., 2009, Hendrickson et al., 2010, Hendrickson et al., 2011, Liu et

al., 2013). For example, rewarding doses of ethanol fail to induce a conditioned place preference in mice lacking the α4 subunit of nAChRs (α4 KO) and low doses of ethanol that cannot conditioned a place preference in WT mice are rewarding in mice expressing hypersensitive α4* nAChRs (Liu et al., 2013). Additionally, varenicline, a FDA approved α4β2* nAChR partial agonist, can reduce ethanol consumption in rodent models and in heavy-drinking smokers, which implicates α4β2* nAChRs as therapeutic targets for ethanol behaviors (Steensland et al., 2007, McKee et al., 2009, Hendrickson et al., 2010). Varenicline was designed to target α4β2* nAChRs, but it is also a partial agonist of α3β2* and α6* nAChRs and a full agonist of α3β4 and α7 subtypes, but with a much lower affinity (Coe et al., 2005, Jorenby et al., 2006, Mihalak et al., 2006). In self-administration studies, knocking out α7 or β2* nAChR is not sufficient to block reductions in ethanol consumption by varenicline, providing evidence of varenicline's promiscuity (Kamens et al., 2010a).

Anesthetic properties of ethanol are also modulated by nAChRs. Nicotine attenuates ataxia induced by ethanol through α4β2* and α7 nAChRs in the cerebellum (Dar et al., 1994, Taslim et al., 2008, Taslim and Saeed Dar, 2011). Mice selectively bred for differences in hypnotic responses to ethanol differentiate in their sensitivity to nicotine's effect on heart rate, locomotor activity, body temperature, and seizures (Heston et al., 1974, De Fiebre et al., 1987). Binding differences to nicotine and α-bungarotoxin in these mouse lines were

measured in the cerebellum, thus implicating both high affinity and α7* nAChRs (De Fiebre et al., 1987). Further studies in these mouse strains found difference in polymorphisms in non-coding and coding gene sequences of the α4 nAChR subunit (Stitzel et al., 2000, Stitzel et al., 2001). Additionally, cerebral inhibition of α4* nAChRs attenuates ataxia induced by anesthetic doses of ethanol (Taslim et al., 2008). Conversely, varenicline increases ataxic and hypnotic effects of ethanol, however it is unclear if this effect is through α4* nAChRs (Kamens et al., 2010b).

To confirm that α4* nAChRs modulate the hypnotic effects of ethanol I measured the latency to the loss of righting reflex (LORR) and duration of the LORR in α4 KO and WT mice. I also tested tolerance to the hypnotic effects of ethanol in these mice by administering hypnotic doses of ethanol for 5 consecutive days. My results show acute effects of ethanol in WT and α4 KO mice were not significantly different. However, after 5 days of ethanol exposure, α4 KO mice were more resistance to the ethanol-induced tolerance measured by the LORR, suggesting that α4* nAChR play a role in ethanol tolerance.

AI.B. Materials and Methods

Animals Drug naïve female α4 nAChR subunit knock-out (α4 KO) mice and wild-type littermates (WT) were used to test the hypnotic effects of ethanol. α4 KO

mice were backcrossed onto the C57BL/6J background for at least 9 generations and genetic engineering of these mice were described by Ross et al. (Ross et al, 2000). In the University of Massachusetts Medical School animal facilities, mice were bred and housed with no more than 5 mice per cage and given food and water *ad libitum* while on 12hr light: 12 hr dark lighting cycle. All experiments were conducted under an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and followed the guideless of the National Research Council (National Research Council, 1996).

Drugs 190 proof absolute anhydrous ethanol (Pharmco-Aaper) was dissolved in 0.9% saline and was administered at a concentration of 4 g/kg via an interperitoneal (i.p.) injection.

Loss of Righting Reflex The loss of righting reflex (LORR) assay is used to study the hypnotic effects of drugs. To avoid variation in LORR experiments from stress of injection and handling, mice were given a saline (i.p.) injection once a day for 3 days prior to each experiment. Additionally, on the day of the experiment, mice were habituated to the room for approximately 40 minutes. The latency to the LORR and duration of the LORR was measured after 4 g/kg ethanol (i.p.). Immediately after ethanol administration mice were continuously placed in the supine position, in a v-shaped trough, until mice were unable to

right themselves within 5 seconds. The time point between the ethanol injection and the LORR was recorded and indicates the latency to the LORR. Time was continuously measured until mice they able to right themselves 3 times within 1 minute, which was deemed the time of recovery. The duration of the LORR was calculated as the recovery time minus the latency to the LORR.

Tolerance to the Ethanol-Induced LORR Ethanol (4 g/kg, i.p.) was administered once a day for 5 consecutive days. LORR latency and duration were measured on days 1, 2 and 5 to assess acute tolerance between days 1 and 2 and chronic tolerance (between days 1 and 5). A difference score measured the change in LORR by subtracting LORR on the day measured from day 1.

Data Analysis Behavioral data were analyzed with an unpaired t-test or two-way multiple comparisons ANOVA as indicated using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

AI.C. Results

To identify a role for α4* nAChRs in the hypnotic response to ethanol, we assessed the loss of righting reflex in α4 KO and WT mice over 5 consecutive ethanol treatments. There was no significant difference in the latency to LORR measured between WT and α4 KO on any of the days tested, suggesting that

initiation of ethanol-induced hypnosis is not modulated by α4* nAChRs (data not shown). After the first exposure to ethanol (Fig. AI-1a) there was a trend, although not statistically significant, for α4 KO mice to have a longer duration of the ethanol-induced LORR. The trend developed into a significant change by day 2 of ethanol administration measured by an unpaired t test ($t=6.620$, $p<0.05$) (Fig. AI-1b). The difference between α4 KO and WT mice was further enhanced by day 5 ($t=3.455$, $p<0.01$) (Fig. AI-1c).

AI.D. Discussion

Varenicline increases duration of ethanol-induced ataxia at high doses, however varenicline is not selective for only α4β2* nAChRs, so the mechanism of its actions is unclear (Kamens et al., 2010b). Despite varenicline's lack of selectivity, I hypothesize that α4* nAChRs modulate the hypnotic effects of alcohol. I evaluated the latency and duration of the ethanol-induced LORR after an acute dose of ethanol in α4 KO and WT mice. Measuring the LORR response after exposing mice to the same dose of ethanol for 5 consecutive days also tested tolerance to the hypnotic effects of ethanol in α4 KO mice and WT. My results indicated that there were no significant differences in the acute effects of ethanol between α4 KO and WT in the LORR duration. However, α4 KO displayed a delayed tolerance to the hypnotic effects after repeated ethanol exposures.

I observed a trend toward an increase in LORR duration in α4 KO compared with WT mice on day 1, but it was not statistically significant, suggesting that α4* nAChR do not have a major role in the modulation of acute ethanol-induced hypnosis. These data on acute ethanol-induced hypnosis were surprising because hypnosis studies in α5, α6, α7, and β2 KO mice reveal these receptor subunits influence acute effects of ethanol hypnosis (Bowers et al., 2005, Kamens et al., 2012, Dawson et al., 2013, Santos et al., 2013). No changes were measured in latency to ethanol-induced LORR on any day (data not shown), which was consistent with these previous studies (Bowers et al., 2005, Kamens et al., 2012, Dawson et al., 2013, Santos et al., 2013). However, reductions in ethanol-induced LORR duration were observed in β2 KO mice and in WT mice challenged with DHβE, an α4β2* nAChR antagonist (Dawson et al., 2013). This response complements increases in ethanol-induced sleep duration by varenicline. Unlike modulation by β2* nAChRs, my study shows that genetic deletion of α4 trends toward an increase in sensitivity toward ethanol-induced sleep duration. The opposing effects in β2 KO and α4 KO mice suggest that separate receptors subtypes modulate these behaviors. For example α3β2 nAChRs have been implicated in ethanol-induced hyperlocomotion and genetic deletion of the α3 nAChR subunit resulted in decreased sensitivity to acute ethanol (Larsson et al., 2002, Kamens et al., 2009).

Ethanol-induced hypnosis studies in α5, α6, and α7 subunit KO mice have reported significantly longer LORR durations (Bowers et al., 2005, Kamens et al., 2012, Santos et al., 2013). α5 or α6 subunits may co-assemble in α4* nAChRs, therefore I expected that α4* nAChRs would also modulate this response. This study suggests that α4* nAChRs do not play a major role in modulating acute hypnotic effects of ethanol and that α5* and α6* nAChR that do not contain the α4 subunit most likely modulate acute hypnotic effects of ethanol (Bowers et al., 2005).

Although α4* nAChR did not play a major role in the acute effects of ethanol-induced hypnosis, there was statistically significant increase in the LORR duration in α4 KO compared to WT mice by day 2. Since the trend on day 1 developed into a statistically significant difference by day 2 and day 5, it is possible that α4* nAChR may play a minor role in the acute effects of ethanol-induced hypnosis. The increased sensitivity to ethanol is not an effect of sensitization since both α4 KO and WT mice develop tolerance to the hypnotic effects of ethanol. WT mice developed a tolerance to ethanol-induced hypnosis by day 2 which decreased further by day 5, however the LORR duration in α4 KO mice did not change on day 2, but was decreased by day 5. This suggests that α4 KO have a delayed tolerance response to the hypnotic effects of ethanol. Since α4 KO mice developed tolerance without α4* nAChRs, it is possible that

there were compensatory changes in the α4 KO mice that may effect this behavior.

Differences in ethanol metabolism could account for differences in ethanol-induced LORR duration. Liu et al. confirmed there were no acute differences in ethanol metabolism between α4 KO and WT mice after 2 g/kg of ethanol (Liu et al., 2013). It is still possible that metabolic changes between α4 KO and WT mice may occur at the higher doses of ethanol, which were used in this study, and after multiple exposures to ethanol. Collins et al. verified that tolerance to multiple exposures of ethanol is an effect of reduced sensitivity in the CNS after chronic ethanol treatments because decreased ethanol-induced sleep time occur despite increased blood ethanol levels (Collins et al., 1988). Although ethanol metabolism still needs to be measured in α4 KO and WTLM after multiple exposures, it is unlikely that there are significant differences that would account for differences in LORR duration between α4 KO and WTLM mice after multiple exposures to ethanol.

This study provides evidence for α4* nAChRs modulation of tolerance to the hypnotic effects of ethanol after repeated exposures, but not to the acute effects of ethanol-induced hypnosis. The mechanisms of alcohol tolerance and hypnosis are poorly understood. This works expands the role of α4* nAChRs in ethanol-

behaviors and adds to the subtypes of nAChRs which modulate hypnotic effects of ethanol.

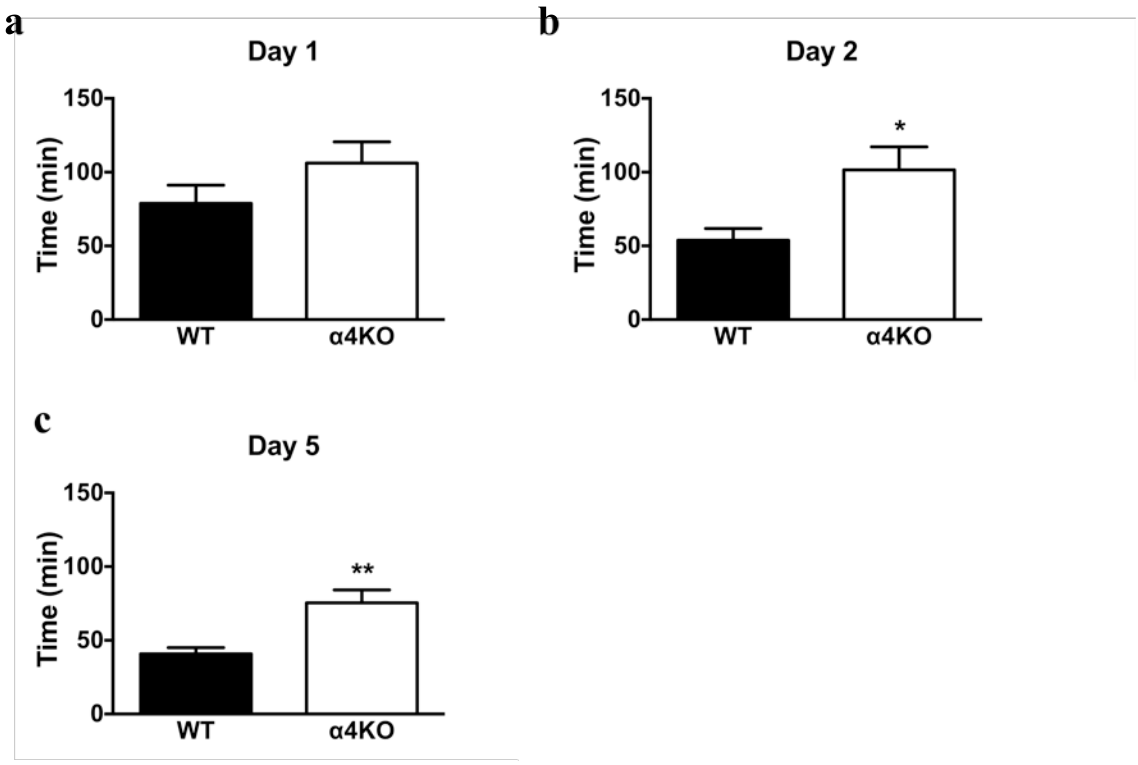

Figure AI-1. α4 KO are more sensitive to the ethanol-induced LORR
Duration of the LORR response to ethanol. LORR duration was measured in WT (n=10) and α4 KO (n=11) after 4 g/kg ethanol (i.p.) on a) day 1 b) day 2 and c) day 5. *p<0.05 and **p<0.01 in unpaired t-test

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